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Effects of washing units of canine red blood cells on storage lesions

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In humans, washing stored blood products prior to transfusion reduces storage lesions and the potential for transfusion reaction, but the effectiveness of washing units of canine whole blood is unknown. The objective of this study was to determine if a manual method of washing of stored whole blood units reduced storage lesions without adversely affecting erythrocytes. Units of canine whole blood were stored for 28 days and manually washed three times with sterile .9% NaCl. Following the first wash, there was a decrease in serum potassium (P<.0001), lactate (P<.0001), pH (P=.0110), pCO_2 (P<.0001), TCO_2 (P<.0001), arachidonic acid (P<.0001), and thromboxane B_2 (P=.0417), and increases in iCa (P=.0494), iMg (P=.0024), MCV (P<.0001), MCHC (P=.0093), RDW (P=.0009), hemoglobin (P=.0011), and MCF (P=.0006). No bacterial growth was identified on the post-transfusion samples. Manual washing of stored blood significantly reduces storage lesions after a single wash and additional washing may cause *in vitro* hemolysis.

DEDICATION

I would like to dedicate this research to my amazing friends and colleagues; Drs. Julianna Frum, Nataly Mamaliger, Katie Cooley-Lock, Erica Burkland, Ryan Gibson, and Chris Tollefson. Their steadfast support over the last four years has been nothing short of amazing. For them, their love, and their support, I have completed this thesis and residency and I owe them all. Thank you all sincerely—this one is for you!

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

Transfusions in Veterinary Medicine

The history of transfusion medicine in dogs dates back to the early 1600s and, in 1665, Richard Lower performed the first canine transfusion when he drew blood from one dog and replaced it with the blood from another dog[1]. After that, it was not until the mid-1900s and the pressure of a world war, that transfusion medicine began to see progress with the development of preservative additives and modern storage blood banking systems[2]. Over the last 40-50 years, veterinary transfusion medicine has followed closely behind human transfusion medicine in efforts to provide the most appropriate blood product to patients while reducing consequences of transfusions[3]. Today, transfusions of whole blood or packed red blood cells (pRBCs) are commonly used in the treatment of life-threatening anemia in critical veterinary patients[4].

Initial efforts to progress transfusion medicine in dogs came about with the standardization of canine blood types based on the presence of inherited erythrocyte cell surface antigens. Detection and standardization efforts in the early 1970s[5,6] resulted in which is now widely recognized as dog erythrocyte antigens. There are currently several recognized classical dog erythrocyte antigens (DEA)s: 1.1, 3, 4, 5, 7, 6 and 8. These antigens vary in their immunogenicity and, thus, some like DEA 1.1 and DEA 7 are more clinically significant while DEA 6 and 8 have no anti-DEA antibodies against them and have been determined to be clinically insignificant[7,8]. DEA 1.1 have the highest incidence rate within the United States and

together make up over 60% of canine blood groups[6,7,9]. Donors that are negative for DEA 1.1, 3, 5, and 7, and positive for DEA 4 are considered to be universal canine blood donors; yet despite this status this blood should not be considered universally safe[8]. Other unique canine blood group systems have been described in veterinary hematology texts, including N-acetyl-neuraminic acid and N-glycolyl-neuraminic acid on RBC membranes of Kai, Kishi, Japanese spaniels, Shiba dogs, and Japanese mongrels[9]. Another blood group identified in Akitas, Shiba, Kishus, Shikous and Japanese mongrels is the D system blood group [9]. A more recently identified DEA-independent Dal erythrocyte antigen has been identified as being absent in Dalmatians, Dobermann pinschers, and Shih Tzus[10]. Despite widely used mechanisms to type and even crossmatch blood prior to transfusion, some recipients still develop life-threatening reactions during and after blood transfusions[11-13]; making it clear that erythrocyte antigens are not the only immunogenic component in blood products.

Transfusion Reactions in Dogs

Although transfusion is a necessary and frequently life-saving therapy, all transfusion recipients, no matter the blood product, remain at risk for the development of adverse events and severe, potentially life-threatening reactions. In veterinary medicine, over 10% of all blood transfusion recipients are reported to develop a transfusion reaction[3,11,14-16].

Transfusion reactions can occur through immunologic and non-immunologic mechanisms[17]. Acute immunologic reactions include the most severe reactions: acute hemolytic transfusion reactions. These occur when the recipient patient has existing antibodies to erythrocyte antigens, and often occur when dogs are transfused mis-matched blood (or when cross-matching was not performed) or had unknowingly been transfused previously allowing for alloimmunization[13,18]. The ensuing reaction develops as a type II hypersensitivity reaction, in

which IgG or IgM antibodies bind donor red cells and activate complement resulting in intravascular hemolysis in a dose-dependent manner[13].

Allergic or type I hypersensitivity reactions can occur when antigens within donor blood interact with IgE or IgG in recipients, resulting in mast cell and basophil degranulation[13]. A more severe version of this reaction can occur when leukocytes within the unit of blood product degranulate and release vasoactive substances that are then transfused into recipients resulting in an anaphylactoid transfusion reaction[13]. Allergic reactions can be very mild, and their true incidence in veterinary medicine is likely unknown, but anaphylactic transfusion reactions can be difficult to distinguish in veterinary medicine from other allergic reactions[13,17,19,20].

One of the most common transfusion reactions to occur in dogs is a febrile, non-hemolytic transfusion reaction[12,13,15]. This reaction is the result of leukocyte antigens present on donor white blood cells and platelets that react with recipient antibodies upon transfusion. This can result in increased concentrations of the cytokines interleukin 1beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor alpha (TNF-α) within the stored unit[12,13], and typically results in an elevated temperature of 1-2°C within 1-2 hours following a transfusion [21].

The last of the acute immunologic reactions is transfusion-associated lung injury (TRALI). This reaction is the result of leukocyte antibodies within the donor plasma against recipient leukocytes causing leukocyte activation, usually within the lungs as a “second hit” after previous priming of the leukocytes within the pulmonary vasculature. Clinical signs with this reaction can occur 1-6 hours after transfusion of a plasma-containing blood product[13], and can be life threatening. The reported incidence of transfusion-associated lung injury in dogs is 3.7%; much lower than the incidence reported in humans[17,22].

Another common transfusion reaction in dogs is transfusion-associated circulatory overload (TACO)[23]. As the name implies, this transfusion reaction occurs when large volumes of products are provided to a patient such that they overwhelm the circulatory system[13,17]. This reaction is not immunogenic in its origin, and is often treated by reducing the rate or the volume of transfusions, and even by adding diuretic therapy[4,13,13].

Non-immunogenic causes of transfusion reactions may occur as well, including transmission of infectious diseases, citrate toxicity, hypothermia, and bacterial contamination/sepsis[13]. Finally, delayed transfusion reactions are also reported in dogs, including delayed hemolytic transfusion reactions, and post-transfusion purpura[13,17].

With the potential for severe reactions, transfusions can carry with them the risk of increasing morbidity and mortality in both veterinary patients and humans, alike. Thus, the focus over the last few decades in transfusion medicine research has been on the identification of causes of transfusion reactions as well as mechanisms for prevention of these reactions. As part of this process, the consequences of blood product storage have come to the forefront. We now recognize that the abnormal conditions created during *ex vivo* storage of units of blood can have a significant impact on the microenvironment of the stored red cells and plasma. In fact, we now know that while erythrocyte, leukocyte, and platelet metabolism is altered during cool storage, metabolic and natural cellular processes continue, and can lead to the development of storage lesions within the stored units of blood. These storage lesions may play a significant role in transfusion reactions and patient outcome, and thus a great deal of human and veterinary research has gone into their identification and prevention.

Storage Lesions

Since the development of preservative products allowing for blood storage, blood products can either be collected and immediately transfused, or stored in order to be readily available for the next recipient. Both storage duration and environment can have a substantial impact on RBC fragility and health. The changes that occur in units of stored blood are known as "storage lesions", and can create biochemical, biomechanical, and immunologic events[24,25] that result in oxidative damage to erythrocytes and metabolic impairments[26]. In human medicine, red cell storage lesions are being increasingly identified in transfusion-related issues[27].

Impaired Erythrocyte Metabolism

Storage causes a decline of erythrocyte 2,3-diphospholgycerate (2,3-DPG), with a decrease to below 54% of prestorage values within just 24 hours of storage of canine blood[28], and a decrease to below detectable limits in human blood products within 14 days for storage at 4^{o}C[29].The depletion of 2,3-DPG promotes the left shift of the oxygen-hemoglobin dissociation curve; thereby increasing the affinity for oxygen to hemoglobin and preventing unloading of oxygen in the tissues: this process has been suggested as a potential cause of increased patient morbidity and mortality post-transfusion[29]. However, this process may primarily be a theoretical consequence, as studies have not supported a significant change in tissue oxygenation post-transfusion[30], and levels of 2,3-DPG are restored to normal within 72 hours of transfusion[31]. Additionally, some veterinary species, like cats, do not depend on 2,3-DPG to increase hemoglobin affinity[32].

Storage also results in a 60% decrease in intracellular RBC ATP after 5 weeks of storage of human blood[29]. The decline in ATP leads a myriad of metabolic derangements and complications within the unit of blood, including oxidation of hemoglobin, proteins, and lipids[26]

that can result in alterations in red cell morphology and deformability[33], increased red cell hemolysis, increased cell free hemoglobin[29], decreased pH with increased lactic and pyruvic acids[34], failure of enzymatic activity, and the formation of reactive oxygen species[24, 35-37]. In particular, Na-K-ATPase in humans is an ATP dependent enzyme that helps to pump potassium into the cell, and sodium out of the cell, and has diminished function under colder temperature or in the ATP-depleted environments created by product storage[38]. In humans, plasma potassium concentrations can increase significantly throughout storage, and can be a critical storage lesion that must be addressed prior to the transfusion of neonates and infants undergoing cardiothoracic surgery or in those receiving massive transfusions[39]. In most dogs, however, the Na-K-ATPase enzyme is not as active, and therefore intraerythrocytic potassium concentrations do not reach near the level as they do in humans[32], and thus the concern for transfusion-induced hyperkalemia is practically non-existent. However, there are several breeds of Japanese and Korean dogs (like Akitas) that have increased activity of Na-K -ATPase[40] whose stored blood product, if these breeds are used as donors, could result in significantly elevated plasma potassium concentrations and may therefore pose a risk during transfusions.

Oxidative Damage to Erythrocytes

Oxidative damage and the exposure of red cells to abnormal levels of oxygen during storage as oxygen seeps into the unit of blood is one of the most important mechanisms of storage lesion development[26]. Oxidation of hemoglobin can result in the release of cell free hemoglobin that can be oxidized and lead to the subsequent development of superoxide and other reactive oxygen species[32]. As ATP and glutathione levels are depleted, these reactive oxygen species can oxidize proteins and lipids and wreak havoc on stored red cells[41]. Oxidized proteins become denatured, aggregate, or precipitate out of solution, resulting in damaged cellular

membranes and cytoskeletons, altered surface proteins, with resultant activation of advanced glycation end products that can be harmful to cells, increase reactive oxygen species, and increase inflammation[26].

Eicosanoids as Storage Lesions

The oxidation of lipids and polyunsaturated fats in stored blood has been investigated by our research group within the last decade. Along with linolenic acid and docosahexaenoic acid, arachidonic acid levels have been documented to increase significantly through human blood storage[42]. Furthermore, eicosanoids like prostaglandin F2α ($PGF_{2\alpha}$), thromboxane B2 (TXB_2), 6-keto-prostaglandin F1α (6-keto-$PGF_{1\alpha}$), leukotriene B4 (LTB_4), hydroxyeicosatetraenoic acids (HETEs), and hydroxyoctadecadienoic acids (HODEs) have been documented to increase during storage of both human and canine blood products[42,43]. These are bioactive molecules derived from arachidonic acid that have been noted to be present at variable levels in donor blood products, but all can increase throughout storage[42] and have been theorized to increase the risk of transfusion reactions[44-46].

Leukocytes within the stored unit of blood are thought to be the primary source of these eicosanoids[41,47]. Cyclooxygenase 1 (COX1) expression on platelets primarily promotes thromboxane formation, while cyclooxygenase (COX2) expression in leukocytes favor the formation of prostaglandins[47,48]. In a study by Fu et al. in 2016, researchers surmised that a lack of prostaglandin detection within stored human blood product was likely due to little to no leukocyte COX2 activity[42]. This can be a source of variability not only from one donor to the next, but also between species. In fact, while the previously mentioned study did not detect levels of any prostaglandin, a different study on murine stored blood not only detected prostaglandins in stored blood but also demonstrated increased levels of many eicosanoids (PGE_2, 5-HETE, 12-

HETE, 15-HETE, 13-HODE and 9-HODE) throughout storage[49]. This highlights the need to further investigate storage lesions within each individual species.

Erythrocytes have been described as a one potential source of eicosanoid synthesis[42,50,51]. RBCs synthesize eicosanoids from the arachidonic acid that is released by phospholipase A2 from the cellular phospholipid membrane[48,52]. Phospholipase A2 can be activated by hormones, cytokines, mechanical injury[53] and even decreased temperatures seen with blood storage[54]. Erythrocytes can also produce HETEs and HODEs through the nonenzymatic lipid peroxidation pathways of arachidonic acid and linolenic acid, respectively[42]. Studies that have removed leukocytes through a process called leukoreduction have further demonstrated increases in eicosanoids, HETEs and HODEs throughout storage, regardless of leukoreduced states, suggesting a role of other remaining cells such as erythrocytes[42].

The full clinical significance of eicosanoids as storage lesions has not been entirely elucidated yet, but there have been human and animal studies demonstrating their importance. In one murine study, stored mouse blood generated increased levels of HETEs and HODEs, and the increase in these eicosanoids was associated with poor recovery in mice post-transfusion[49]. Additionally, arachidonic acid and HETEs have been implicated as the "second hit" in the two-hit model for TRALI[41]. Other clinical consequences of exposure to increased eicosanoids include the immunomodulatory effects of PGE_2[55], bolstering of systemic inflammatory response syndrome (SIRS) responses by leukotrienes and thromboxanes[56-60], and leukocyte and platelet activation by thromboxanes[61,62]. Thromboxanes and HETEs mediate vasoconstriction and, in this way, transfusion of eicosanoids have been theorized to generate vascular effects on recipients, but this has not been clinically demonstrated[63,64]. Further investigation into the effects of eicosanoids as storage lesions and their physiologic effects on transfusion recipients is needed.

Microparticles & Hemolysis

Oxidation of erythrocytic lipids does not just lead to eicosanoid formation; it can also result in the vesiculation of red cell membrane phospholipids, including exposed phosphatidylserine, leading to increased microparticles within units of stored blood cells[52]. The budding of the membrane to form microparticles *in vivo* is a protective mechanism for erythrocytes, as the microparticles would be subsequently removed from circulation by the reticuloendothelial system[65]. Depletion in ATP availability no longer prevents cell surface expression of phosphatidylserine, a surface marker that promotes macrophage clearance of red cells post-transfusion[32,41,66,67]. In storage, microparticle formation can have significant consequences. Microparticles can lead to procoagulant activity by allowing for exposure of negatively charged phosphatidylserine that promotes thrombin formation through tenase and prothrombinase activity[68]. The microparticles can also have procoagulant activity through their expression of tissue factor[69] and increasing adherence of leukocytes to vascular endothelium[65], and the microparticles can also be pro-inflammatory once transfused by promoting cytokine release[70]. This has prompted research in the veterinary fields into methods for microparticle detection and easier ways for quantification[71].

Some of the earliest storage lesions described were progressive changes in RBC shape, with development of echinocytes and then ultimately irreversible change to spheroechinocytes or spherocytes[33,72,73]. Additionally, as cell health declines in stored units, there is increased risk of erythrocyte lysis and release of cell free hemoglobin[29,33]. This phenomenon can pose a significant health risk to the patient: as an example, Patterson *et al*. reported the death of three of four dogs that developed an acute hemolytic reaction due to inappropriate storage of red blood cell products[25].Transfusion of stored blood with high concentrations of cell free hemoglobin can

have detrimental physiologic effects on the recipient, not only due to transfusion reactions but also through direct effects on vascular nitric oxide availability and bacterial metabolism. Nitric oxide (NO) is produced *in vivo* by vascular endothelial cells, and its primary role is local vasodilation[74]. Other important roles of NO include inhibition of platelet aggregation and expression of adhesion molecules, demonstrating that scavenging of NO could result in increased risk of thromboembolic events, increased leukocyte adhesion and diapedesis, and increased endothelial permeability[74]. When stored blood with increased concentrations of cell free hemoglobin are transfused into patients, the natural buffering mechanism for excess hemoglobin, haptoglobin, becomes rapidly overwhelmed. Residual hemoglobin is instead able to interact with and scavenge nitric oxide resulting in vascular dysfunction within the recipient[74]. Consequences of this depletion of NO by cell free hemoglobin were previously demonstrated in a study by Solomon et al. that showed significant vasoconstriction and improved patient outcome in models of canine hypovolemic shock with dogs that had increased levels of NTIB and cell free hemoglobin[75]. In a veterinary review on storage lesions, a large focus was on the consequences of free hemoglobin or iron, and on their scavenging effects[24].

Other Storage Lesions

In humans, it has been shown that, as well as contributing to red cell fragility, the act of storing blood products leads to the accumulation of cytokines such as IL-1β, IL-6, and IL-8[44,76]. Accumulation of these molecules is believed to significantly increase the risk of transfusion reactions[45]. Storage of canine blood products appears to lead to accumulation of the same classes of molecules that are observed with stored human products. Recent work by Corsi and others documented a significant increase in the pro-inflammatory cytokine IL-8 in units of stored canine pRBCs[46].

A 2001 study investigated the accumulation of ammonia in canine packed red blood cells, and found that ammonia concentrations increased during storage[77]. The ammonia was suspected to be secondary to the deamination of amino acids within the red cell units, as well as breakdown of purines that are components of the preservative solutions[77]. The dogs that were transfused pRBCs with increased ammonia levels, but did not have increased levels of ammonia in circulation post-transfusion[77]. The authors of this study suggested that this indicated that healthy dogs can metabolize this storage lesion, but that it was possible that dogs with liver dysfunction could be negatively affected by the excess ammonia source in stored blood products[77].

Documented Consequences of Storage Lesions

The extent of storage lesion development is still being heavily researched, as are the consequences of those storage lesions in patients. There is wide debate on whether the transfusion of older blood increases morbidity in transfusion recipients. A retrospective evaluation of red cell product age on transfusion-related complications in dogs revealed an increased risk of hemolysis (odds ratio 1.11) with each additional day of age of storage[78]. Yet, in this study, and another study, transfusion of dogs with older blood products was not associated with increased mortality[78,79], although it is possible that the study performed by Holowaychuk et al was underpowered[79]. In human pediatric cardiac surgery patients, red cell storage age was also not associated with adverse events[80]. Additionally, in one study of hemorrhagic shock in dogs, transfusion of older blood was actually associated with an improved outcome, with reduced mortality rates and decreased norepinephrine requirements[75]. The benefits the use of older blood in this study were attributed to the vasoconstrictive effects of cell free hemoglobin through the scavenging of NO[75]. Certainly, it is possible that in some circumstances, transfusion of older

blood with associated storage lesions may prove beneficial or even lifesaving in patients. Further investigation into this area is recommended.

There are, however, an abundance of literature reviews highlighting storage lesions in humans and dogs, and the association between these storage lesions and negative consequences or outcomes[24,26,81-83]. The length of storage has been demonstrated to contribute to a negative case outcome in dogs with hemolysis or immune-mediated hemolytic anemia[15]. Human trauma patients have an increased risk of multiple organ failure when receiving blood that is stored for longer than 14 days (odds ratio 1.16), with even greater risk of multiple organ failure with stored blood that is older than 21 days (odds ratio 1.22)[84]. In a study using canine models of pneumonia, researchers transfused dogs with blood of variable storage age, and found that transfused dogs with pneumonia had increased cell free hemoglobin, increased free iron in the form of non-transferrin bound iron (NTBI), and decreased haptoglobin levels if they were transfused with older stored blood compared to those that were transfused with fresh blood[85]. Additionally, those dogs transfused with older blood developed *in vivo* hemolysis of transfused blood, had increased mortality rates, and worsening lung damage on histopathology[85], and the authors of this study proposed that increased free iron likely provided a critical nutrient for bacterial growth, allowing for pneumonia to worsen in these patients. Additionally, scavenging of NO can lead to profound vasoconstriction that results in systemic and pulmonic hypertension, induced ischemia, and vascular endothelial injury with the transfusion of older blood[85,86]. A second study was performed by the same research group, using the same canine model in which dogs with pneumonia were challenged with bacteria after receiving transfusions of either old stored blood or fresh blood[87]. In this second study, the researchers found not only the expected increase in cell free hemoglobin in animals that received older blood, but also a more rapid and greater decline

in NTBI and worsening sepsis[87]. In a study using autologous transfusions in dogs, dogs that were transfused older blood had elevated total iron binding capacity, labile iron hemoglobin and ferritin, with concurrent decreases in transferrin and haptoglobin[88]. In general, increasing iron availability by any means to anemic dogs with infections has been demonstrated to increase morbidity: anemic dogs exhibited increased shock, increased lung injury and increased NTBI after intravenous iron was supplemented in dogs with experimental bacterial pneumonia[89].

Overall, it is becoming increasingly clear that the length of storage of blood product increases the degree of storage lesions, thus potentially increasing the risks associated with transfusion, at least in some clinical circumstances such as hemolysis and pneumonia.

Current Methods to Reduce Storage Lesions

Several methods have been used to reduce the accumulation of storage lesions, improve erythrocyte health, prolong RBC survival in the recipient, and reduce the risk and severity of transfusion reactions. Advancements in cell preservative solutions and additives[90], transfusion techniques[91,92], and ideal storage temperatures[93] have improved erythrocyte health and prolonged the safe duration of storage, but have not completely eliminated the development of storage lesions[26].

Leukoreduction and platelet depletion filters to extract leukocytes and platelets from the unit prior to storage can be utilized in an attempt to prevent these cells from influencing the microenvironment within the unit by producing and releasing inflammatory mediators during storage[94,95]. Leukoreduction has the proven benefits of reducing storage lesions including cytokines[46,76,96] and microparticles[66] by removing leukocytes and platelets that can retain some level of activity within stored units of blood and that thereby may contribute to storage lesion development. Compared to dogs that receive leukoreduced blood, dogs that receive non-

leukoreduced blood have increased total white blood cell counts 2-24 hours after transfusion[97,98], with increased segmented neutrophils, increased fibrinogen levels, and elevated C reactive protein[98].

Leukoreduction is not without its limitations. In dogs, pre-storage leukoreduction and platelet depletion causes an immediate increase in concentrations of thromboxane B_2 and $PGF_{2\alpha}$[99] and, in humans, leukoreduction results in an immediate increase in supernatant potassium levels that may be associated with platelet activation or hemolysis[100] as well as an increase in IL-6[45]. Additionally, in dogs, leukoreduction does not prevent the accumulation 6-keto-$PGF_{2\alpha}$[99] and, in humans undergoing radical prostatectomies, other eicosanoids like PGE_2, TXB_2, and the cytokine IL6 were not reduced by leukoreduction[45]. Additionally, leukoreduction filters do not prevent the survival of some organisms, such as *Rickettsia conorii*[101], in experimentally infected canine blood products. In healthy dogs regardless of their leukoreduced status, transfusion of older blood products still induces an inflammatory response as characterized by increased monocyte chemoattractant protein 1, increased neutrophil counts, decreased platelets, increased NTBI, and evidence of intravascular hemolysis[102].

Rejuvenation techniques that are meant to improve red cell health just prior to transfusion have shown some promise[103]. This technique involves storing blood in solutions rich in inosine or adenine to help restore ATP and 2,3-diphosphoglycerate concentrations within the units, and then removing the solution and remaining plasma through a washing step prior to transfusion[103]. Transfusions of rejuvenated red cells have been shown to be effective in reducing cell free hemoglobin, leukocyte sequestration, lung injury, kidney injury, and endothelial dysfunction in pigs transfused stored blood[103].

An alternative method evaluated for the potential to reduce storage lesions or transfusion reactions is irradiation and UV-irradiation of canine units of blood products[104,105]. Irradiation, however, results in increased levels of potassium, lactate, and cell free hemoglobin, with concurrent decrease in pH[104]. Irradiation has also been associated with increased IL-8 by day 14 of storage post-radiation in canine whole blood[96]. Long-term storage of canine blood products by freezing has also been reported[106,107] and, in a recent study, freezing erythrocytes showed no negative effect on 2,3-DPG, ATP, free hemoglobin, sodium, potassium, and hemograms[106].

With all these different reported methodologies, however, units of stored blood still had storage lesions such as vasoactive molecules that may be transfused into the recipient. An alternative methodology that would be expected to lead to the near total elimination of these vasoactive molecules, such as red blood cell washing immediately prior to transfusion, has the potential to significantly reduce the risk of transfusion reactions.

Red Cell Washing

Introduction to Washing

One method of reducing storage lesions is washing the units of red cells prior to transfusion. Washing of red blood cells prior to transfusion is a technique that effectively removes between 95 and 99.9% of the plasma/supernatant within the unit, including plasma components such as proteins, electrolytes (including potassium), microparticles, antibodies, cellular debris, and other storage lesions that have accumulated in the unit during storage[108]. The process can be performed through manual washing techniques, or through the use of automated washing devices[109,110]. There have been studies in human medicine that have investigated the efficacy of manual versus automated techniques, and have found that manual techniques do a better job at removing plasma protein levels and clearing residual plasma[110,111]. Additional

exploration into the washing of human blood products has evaluated whether it is better to perform the washing just before transfusion ("in the operating room") or at blood storage facilities ("in the blood bank"), and confirmed greater reduction of storage lesions with washing as close to administration as possible (that is, in the operating room) because of a greater ability to reduce cell free hemoglobin and lactate dehydrogenase levels[109]. A proof-of-concept study has developed a novel technique that washes red cells bedside as the transfusion occurs[112], but this technique will require further development before it is readily available in human medicine, let alone in veterinary medicine.

Washing Media

One final area being investigated in red cell washing is an effort to identify the ideal washing medium that supports red cell health[111,113,114]. Studies have investigated the use of saline-adenine-glucose-mannitol (SAGM), 0.9% saline, saline-glucose mixtures, PlasmaLyte®, 5% albumin products, and deglycerolization media, all with variable results, and no cross-over in interpretation between studies can be gleaned[111,113,114]. Specifically, when compared to saline in automated washers, PlasmaLyte® resulted in higher levels of potassium and lower levels of sodium within the human units of blood[114]. In a 3-way comparison, SAGM resulted in less hemolysis and decreased microparticles/ microvesicles compared to saline or saline glucose mixtures as washing media[111].

Storage Lesion Reduction with Washing

Washing red cells has been proven to reduce storage lesions such as cell free hemoglobin[87,115], potassium[39,103,116-119], IgA, total protein, lactate, cytokines, cell surface proteins like E-selectin and CD106[116], and eicosanoids within the red cell supernatant[42]. The use of

washed RBCs has reduced transfusion reactions[86] and decreased the risk of transfusion-associated lung injury in humans[120]. Cholette *et al.* showed that washing of red cell components prior to transfusion decreased systemic inflammatory mediators, IL-6, IL-6:IL-10 ratios, and C reactive protein levels in children undergoing cardiac surgery, and resulted in a need for a lesser number of transfusions[121].

Currently, the effects of pre-transfusion washing of blood products in dogs is mostly unknown. However, using a canine pneumonia model, the use of washed pRBCs prior to transfusion reduced multiple organ injury and improved survival rates compared to non-washed blood products[115], suggesting that pre-transfusion washing may benefit dogs receiving blood products.

Although the use of washed blood products would be expected to decrease storage lesions and reduce the risk of transfusion reactions, the simple act of washing itself could cause some unintended consequences. One such consequence is erythrocyte swelling. The cellular swelling during washing is widely considered to be secondary to build up of osmotically active substances (such as lactate, chloride, and 2,3-DPG) intracellularly[32,34]. These osmotically active substances develop during storage and, after washing, the extracellular osmolality decreases significantly depending on the washing media used[120,122], potentially resulting in an intracellular shift of fluid. Additionally, the storage-related dysfunction of Na-K-ATPase that causes an increase concentration of intracellular sodium may also lead to an osmotic pull of water into the RBC and cell swelling[24,38,123].

Increased MCV due to erythrocyte swelling shortens the lifespan of erythrocytes and increases osmotic fragility due to the decreased surface area to volume ratio and decreased deformability[34,123]. Washing RBCs could, for example, increase RBC fragility and lead to

hemolysis during or after transfusion[108]. Grabmer *et al.,* for example, reported an increase in hemolysis following washing, but the degree of hemolysis remained less than 0.8%, the maximum level of accepted hemolysis recommended by the Council of Europe[116]. Loh *et al.* also demonstrated a minor increase in hemolysis and a minor loss of red cells during washing[124]. Finally, Weisbach *et al*. showed a significant loss of 2,3-DPG levels in washed RBCs compared to non-washed RBCs, especially in older erythrocytes[125].

The normal plasma osmolality in dogs is approximately 300mOsm/L and with the addition of the storage media, and then subsequently throughout storage, plasma osmolality would be expected to increase, potentially resulting in echinocyte formation. Washing media like those widely used in human and veterinary medicine often have different osmolalities than that of normal canine plasma. Lactated Ringer's solution has an osmolality of 273 mOsm/L while normal saline has an osmolality of 308 mOsm/L. As mentioned above, washing with normal saline can results in cellular swelling of stored erythrocytes and so researchers have explored washing human red cells with solutions containing dextrose, mannitol, or albumin that exhibit osmotic or colloidal oncotic effects[111,113,122,125]. Findings from those studies revealed improve red cell morphology as evidenced by normalized MCV and increased MCHC, as well as decreased erythrocyte water content and improved cell health[111,113,122,125]. No studies in veterinary patients have looked at the optimal washing media for canine stored red cells.

Methods for Erythrocyte Washing

Washing red cells within blood units can be performed by multiple techniques that include an automated closed system, centrifugation techniques, or mechanical washing with an open system. All these techniques suspend the red cells in saline or other isotonic/ preservative fluids[108]. Automated closed systems that wash units of blood prior to transfusion can be

expensive, and are not readily available in veterinary hospitals. Therefore, if washing of blood products is going to be utilized in veterinary clinics, manual techniques will almost certainly be the most common method used. Because manual washing techniques are largely unexplored in veterinary medicine, and methodology must be extrapolated from human medicine, the most appropriate protocols for use in dogs are unknown.

Currently, it is unknown how many washes are needed for canine blood units, the ideal washing solution, the appropriate centrifugation speed and time, or even the optimal time allowed for storage after washing. In humans, there are several washing techniques that have been investigated for the purposes of comparing automated washing versus manual washing in terms of efficacy of reducing storage lesions[111,114], but no such studies have been reported in dogs. The general techniques for manual washing of human stored red cells involve performing three washes with the wash media[124,125], but reported studies of washing canine red cells have used automated washers[86,115,126], and no investigation has therefore looked into the best number of manual washes for canine stored red cells. Even the centrifugation speed and time for manual washing techniques is variable, with reported speeds between 2900×g to 4950×g, and times ranging from 5 to 15 minutes for each wash step[111,124,125]. There is still a great deal more to uncover in veterinary transfusion medicine with regards to the washing of stored canine red cell units.

The use of pre-transfusion washed red blood cell products has the potential to reduce storage lesions and prevent transfusion reactions in veterinary patients, especially, based on the human experience, in smaller dogs and cats, neonates, and patients that require multiple or massive transfusions. The long-term goal of our research group is to establish blood transfusion protocols that allow veterinarians to select safer blood products and potentially minimize the frequency

and severity of transfusion reactions. The objectives of the study described in this thesis are to determine if a manual method of washing units of canine whole blood can be performed without causing adverse effects to erythrocytes, and to determine if this technique will effectively reduce storage lesions, including levels of vasoactive prostaglandins and leukotrienes, that accumulate in blood products during storage and transfusion. The study hypotheses were that the washing protocol could be performed without causing significant damage to the washed erythrocytes, and that a single wash could significantly reduce the amount of vasoactive substances and other potentially harmful molecules that accumulate in units of canine whole blood. Because veterinarians use stored blood products more frequently than fresh blood as a life-saving therapy in critical patients[127], the development of a simple manual washing protocol that can safely reduce the risk of transfusion reactions has the potential to have an immediate impact on the clinical use of stored blood products, and to assist clinicians with therapeutic decision-making when treating patients in need of a blood transfusion.

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CHAPTER II

EFFECTS OF WASHING UNITS OF CANINE RED BLOOD CELLS ON STORAGE LESIONS

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Introduction

Stored blood products like whole blood and packed red blood cells (pRBC) are commonly used in veterinary medicine. Both storage duration and environment can have a substantial impact on red blood cell (RBC) fragility, leading to both *in vitro* (pre-transfusion) and *in vivo* (post-transfusion) hemolysis. The changes that occur within units of stored blood, known as storage lesions, can create biochemical, biomechanical, and immunologic events that lead to *in vitro* and *in vivo* hemolysis[1]. Prolonged storage can cause oxidation of proteins and lipids, and changes in RBC shape, and decrease the circulating survival time of RBCs post-transfusion. In humans, the accumulation of vasoactive and pro-inflammatory molecules, including cytokines and arachidonic acid (AA)-derived eicosanoids, and increased RBC fragility[2-4] can potentially increase the risk of transfusion reactions[5].

Several methods have been used to reduce the accumulation of storage lesions, improve erythrocyte health, prolong RBC survival in the recipient, and reduce the risk and severity of transfusion reactions. Pre-storage leukoreduction and platelet depletion decrease many

inflammatory mediators and storage lesions in both humans and dogs[2,6-13]. However, pre-storage leukoreduction and platelet depletion causes an immediate increase in concentrations of TXB_2 and doesn't eliminate the accumulation of 6-keto-$PGF_{2\alpha}$in units of canine whole blood[8]. Irradiation of stored canine pRBCs has been used to inactivate lymphocytes, but this process had minimal impact on storage lesion development[14]. Advancements in cell preservative solutions have improved erythrocyte health and prolonged storage durations but have not eliminated storage lesions[15].

The use of a pre-transfusion washing technique can remove up to 95% of the plasma/supernatant within the unit, including proteins, electrolytes, cytokines, microparticles, antibodies, cellular debris, and other storage lesions[10,16-18]. In humans, the use of washed blood products prior to transfusion reduces transfusion reactions, risk of transfusion-associated lung injury, and hyperkalemia in infant transfusion[19-22]. Although washing of blood products would be expected to reduce the accumulation of storage lesions and risk of transfusion reactions, the act of washing could cause some unintended consequences, including increased RBC fragility and hemolysis[10,16,17,23].

There are multiple techniques that can be used to wash blood products, including use of an automated closed system, centrifugation techniques, or mechanical washing with an open system. These techniques use saline or other isotonic/preservative fluids to eliminate storage lesions while maintaining RBC health[16]. Automated closed systems can be expensive, and are not readily available in veterinary hospitals. Therefore, if washing of blood products is going to be utilized in veterinary clinics, manual techniques would probably be the most commonly used method. In veterinary medicine, the most effective manual washing technique, which eliminates storage lesions and perseveres red blood cell health, is unknown.

The objective of this study was to determine if a manual method of washing can effectively reduce storage lesions that have accumulated in units of stored canine whole blood without causing adverse effects to erythrocytes. Our hypotheses were that washing of post-storage units of canine whole blood 1.) would decrease measurable storage lesions prior to a simulated transfusion, with no subsequent increase in lesions over the duration of a simulated transfusion, 2.) would cause minimal erythrocyte damage, evaluated by hemolysis, morphology, and function, and 3.) could be performed in a sterile fashion.

Materials and Methods

Animals

Eight healthy adult research dogs, 5 intact females and 3 castrated males, were used in this study. The mean age of the dogs was 5.1 years (range, 3.5-6 years) and the mean weight was 27.2 kg (range, 24.6-36.1 kg). The dogs were deemed healthy based on no abnormalities on physical examination, CBC (including manual platelet count), serum biochemistry, urinalysis, and heartworm and tick-borne disease testing. The dogs were not exposed to any medications or vaccines for at least 2 weeks prior to the initiation of the study. A sample size calculation was performed using results from previous studies[10,20,24] performed in humans. The calculation was performed based on levels of lactate, potassium, and glucose in pre- and post-washed blood products. Based on these results, 8 dogs were calculated to be needed to have 80% power to find a 25% reduction in storage lesions with an alpha of 0.05. Animal use was approved by the Mississippi State University College of Veterinary Medicine Institutional Animal Care and Use Committee and was in compliance with the requirements of the American Association of Accreditation of Laboratory Animal Care.

Donation and washing technique

Each dog underwent standard blood donation. Briefly, the donors were sedated with acepromazine[1] (0.02mg/kg IV) and butorphanol[2] (0.1mg/kg IV) and positioned in either right or left lateral recumbency. The hair overlaying the right jugular vein was clipped and the skin was aseptically cleaned. A 16-gauge needle was inserted into the jugular vein and, under negative pressure, approximately 450 ml of blood was collected directly into a standard blood banking bag containing citrate phosphate dextrose adenine solution (Teruflex blood bag system, Terumo, Tokyo, Japan). The collection tubing was then stripped to allow for adequate mixing of collected blood with storage bag preservative and then blood was allowed to fill the line again so that a whole blood sample could then be obtained from the connecting tubing prior to storage (*Pre-storage*). There were no adverse events detected in the dogs during or after blood collection. All units were stored vertically at 4°C in a dedicated refrigerator. After 28 days of storage, the units were removed from refrigeration and gently rocked for 1 minute. A sterile blood spike was inserted into the unit and a blood sample was collected (*Post-storage*).

Each unit was manually washed using a protocol previously used in human medicine[23]. Briefly, following collection of the post-storage sample, each unit was centrifuged for 10 minutes at 5°C and 4000×g. A syringe was attached to the blood spike, and the plasma was removed and discarded. The unit was resuspended with 250 ml of cold 0.9% sterile NaCl (Baxter, Copperfield, IL, USA), gently rocked for 1 minute, and a blood sample was collected (*Wash 1*). This centrifugation process was repeated until a total of 3 washes were performed.

[1] Aceprojet, acepromazine maleate injection, USP (10mg/ml) (Henry Schein Inc.; Dublin, OH, USA)
[2] Torbugesic-SA® IV, butorphanol tartrate injection (10mg/ml) (Zoetis; Kalamazoo, MI, USA)

Samples were collected at the completion of each wash step (*Wash 2* and *Wash 3*). After the third wash, the units were centrifuged, the wash saline was removed and 100ml of cold sterile saline was added to the units. To simulate a transfusion of pRBCs, the units remained at room temperature (25°C) for 5 hours. Following the simulated transfusion, the units were gently rocked for 1 minute and a final sample was collected (*Post-transfusion*).

Blood gas analysis

Blood gas analysis (Stat Profile Prime Plus Critical Care Analyzer, NOVA biomedical, Waltham, MA) was performed within 30 minutes of sample collection, except for one post-transfusion sample that was analyzed 7 hours after collection due to instrument malfunction. If the blood gas analyzer was unavailable, a blood lactate (Lactate Plus, NOVA Biomedical, Waltham, MA) and blood glucose (AlphTrak-2 glucometer, Zoetis, Kalamazoo, MI) were preformed separately. The blood gas analyzer included the following parameters: pH, pCO2, pO2, sO2, Na, K, Cl, iCa, iMg, glucose, lactate, methemoglobin (MetHgb), total bilirubin (Tbili), cell free hemoglobin (HgbF), and TCO_2.

Erythrocyte osmotic fragility

Erythrocyte osmotic fragility was completed within 6 hours of sample collection using a previously described dilution technique[25-26]. Briefly, a phosphate-buffered 10% NaCl stock solution was prepared and diluted with distilled water to create a 1% NaCl working solution. For each blood sample, serial dilutions of the working solution (5 ml) were added to 16 tubes to create final concentrations of 0.0% to 0.85% NaCl (increments of 0.05% NaCl). From each

sample, 20 µL was added to each tube, gently mixed, and remained undisturbed at room temperature for 30 minutes. Samples were centrifuged at 2,000×*g* for 10 minutes, and the optical density of the supernatant was measured in triplicate at a wavelength of 540 nm by use of a plate reader (SpectraMax M5 multimode microplate reader, Molecular Devices LLC, Sunnyvale, Calif.). The percent hemolysis was calculated by dividing the optical density of the blood samples at each NaCl concentration by the optical density of the blood sample at 0.0% NaCl. The mean corpuscular fragility (MCF) was calculated to determine which NaCl concentration was associated with 50% hemolysis.

CBC & erythrocyte morphology

A CBC was performed within 4 hours of collection using an automated hematology analyzer (Cell-dyn 3700, Abbott Laboratories, Taguig City, Philippines). The analyzer provided the following parameters: RBC count, WBC count, platelet count, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), and hemoglobin (Hgb).

Erythrocyte morphology was evaluated by a blinded board-certified veterinary clinical pathologist (MLW). For each blood sample, a blood film was prepared with a standard Wright's stain and 10 high-power fields (hpf; 1,000× oil magnification) were evaluated. Microscopic fields were scanned for abnormal RBCs (eg, echinocytes, keratocytes, acanthocytes, schistocytes, and target cells) and the frequency of abnormal cells was scored using a subjective scale as rare (1 to 10 abnormal cells/hpf), few (11 to 50 abnormal cells/hpf), moderate (51 to 100 abnormal cells/hpf), and many (> 100 abnormal cells/hpf).

Aerobic and anaerobic bacterial culture

Using blood-agar plates, aerobic and anaerobic cultures were performed on the *Post-storage* and *Post-transfusion* samples. Samples were considered aseptic if no bacterial growth was detected after 48 hours of observation. If bacterial growth was detected, further identification was performed to determine the species of the isolated bacterial colony.

Eicosanoid analysis

Following collection, blood samples were centrifuged at 1800×g for 8 minutes at 25°C, and the supernatant was frozen in liquid nitrogen and stored at -80°C for later analysis. Using a previously established liquid-chromatography mass spectrometry technique[8,27], with modifications to the sample workup procedure described below, the concentrations of AA, prostaglandin F_{2a} ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), thromboxane B_2 (TXB_2, a stable metabolite of thromboxane A_2), 6-keto-$PGF_{1\alpha}$ (a stable, hydrolyzed product of prostacyclin), hydroxyeicosatetraenoic acid (HETE), and hydroxyoctadecadienoic acid (HODE) were measured.

For analysis, deuterated internal standards were added to the thawed plasma samples in two volumes of ice-cold 1:1 (v/v) methanol/acetonitrile followed by vortex mixing. After chilling at -20°C for 30 min, the samples were centrifuged at 16,100xg for 10 min at 4°C to remove precipitated protein and the organic solvents in the supernatants removed in a speed vac centrifuge. The eicosanoids in the concentrated samples were extracted by using a C18 SepPak column (HyperSep Retain PEP 60 mg, 1 mL, Thermo Fisher Scientific, Waltham, MA) and eluted in methanol. The samples were dried under nitrogen gas, resolubilized in 100 µL of methanol, and 10 µL injected onto a Waters C18 column (1.7 µm particle size, 100mm x 2.1 mm

internal diameter) (Acquity UPLC BEH C18 column, Waters Corporation, Milford, MA). The analytes were eluted with a gradient program and introduced into a mass spectrometer (TSQ Quantum Access Max, Thermo Fisher Scientific Inc). The concentration of each eicosanoid was determined by measuring the area under each chromatographic peak and comparing this result to the area under the chromatographic peak for the internal standard. The eicosanoid concentrations were normalized to the volume of plasma used for analysis and expressed as pmol/mL plasma. The estimated limits of detection were between 0.1 and 10 nM.

Statistical Analysis

Descriptive and inferential statistics were performed using commercially available statistic software (SAS for Windows v. 9.4, SAS Institute, Inc. Cary, NC). Impact of stored blood washing on outcomes of blood gas values, CBC values, and eicosanoid concentrations was initially assessed using a generalized linear mixed model using PROC MIXED, however models did not meet the assumptions of homoscedasticity and normality of the residuals. Consequently, a method similar to the non-parametric Friedman's test was used. Outcomes were first ranked by dog using PROC RANK, then analyzed by linear models using PROC MIXED. Dog identity and sample were included as fixed effects in each model. The LSMESTIMATE statement was used to compare the following samples in the washing protocol using a simulated adjustment method for multiple comparisons: Pre-storage vs. Post-storage, Pre-storage vs. Wash 1; Pre-storage vs. Wash 2; Pre-storage vs. Wash 3; Post-storage vs. Wash 1; Post-storage vs. Wash 2; Post-storage vs. Wash 3; Post-storage vs. Post-transfusion; Wash 1 vs. Wash 2; Wash 1 vs. Wash 3; Wash 2 vs. Wash 3; Wash 3 vs. Post-transfusion. The simulate option in LSMESTIMATE statement was

used to adjust p-values for multiple comparisons among sample times. An alpha level of 0.05 was used to determine statistical significance.

Results

Blood gas analysis

Due to malfunctions with the analyzer, post-storage, Wash 1, Wash 2, Wash 3, and post-transfusion samples were not able to be completely evaluated in one unit of blood and data for these samples only consisted of blood glucose and lactate measurements for all but the pre-storage sample.

The results for glucose, lactate, potassium, sodium, iCa, and iMg are represented in Figure 2.1 and the remaining blood gas results are listed in Table 2.1. There was a significant ($p<0.0001$) increase in lactate after storage, but the lactate decreased following each wash (Wash 1, $p=0.0016$; Wash 2, $p=0.0016$; Wash 3, $p<0.0001$), but increased significantly ($p=0.0003$) during the simulated transfusion. There was a significant decrease in glucose concentration ($p<0.0001$) during storage and after each wash (Wash 1, $p<0.0001$; Wash 2, $p<0.0001$; Wash 3, $p<0.0006$). There was no change ($p=0.9876$) in glucose concentration following the simulated transfusion.

The potassium concentration increased ($p=0.0075$) during storage, but decreased significantly (Wash 1, $p<0.0001$; Wash 2, $p=0.0006$; Wash 3, $p=0.0006$) following each wash. There was no change ($p=0.6917$) in potassium concentration following the simulated transfusion. The sodium concentration increased ($p=0.0159$) during storage, but decreased significantly (Wash 1, $p<0.0001$; Wash 2, $p<0.0001$; Wash 3, $p<0.0001$) following each wash. There was no difference ($p=0.1063$) in sodium concentration between the Pre-storage and Wash 1 samples.

The chloride concentration increased following each wash (Wash 1, $p<0.0002$; Wash 2, $p<0.0001$; Wash 3, $p<0.0001$). There was no change in the ionized calcium ($p=1.000$) and ionized magnesium ($p=1.000$) during storage, but there was a progressive increase in both parameters following each wash and simulated transfusion.

CBC & erythrocyte morphology

The CBC results are summarized in Table 2.2. There were no statistically significant changes in red blood cell parameters between Pre- and Post-storage samples. There was a significant increase in RBC count ($p=0.0017$), hematocrit ($p=0.0006$), hemoglobin concentration ($p=0.0023$), and RDW ($p=0.044$) between Wash 3 and the simulated transfusion. The hematocrit also increased, compared to Pre-storage, following Wash 1 ($p=0.0011$) and Wash 2 ($p=0.0328$).

The MCV, MCHC, and RDW results are presented in Figure 2.2. Compared to Pre- and Post-storage samples, there was a significant increase in MCV after each wash, but there no differences between each wash. The MCV was significantly ($P< .0001$) greater following the simulated transfusion compared to Post-storage samples. Compared to Pre- and Post-storage samples, there was a significant decrease in MCHC after each wash, but not between individual washes. Compared to Post-storage samples, there was a significant ($P< .0001$) decrease in the MCHC after the simulated transfusion. Compared to Pre-storage values, the RDW increased after each wash (Wash 1, $p=0.0009$; Wash 2, $p<0.0001$; Wash 3, $p=0.0002$), but not between each wash. When compared to Post-storage values, there was no change in RDW following Wash 1 ($p=0.135$), but there was an increase following Wash 2 ($p=0.0093$), Wash 3 ($p=0.0128$), and the simulated transfusion ($p<0.0001$).

Compared to Pre- and Post-storage samples, there was a significant decrease in WBC count following all three washes. There was a progressive decrease ($p=0.035$) in WBC count

between the first 2 washes, but there was no difference ($p=0.927$) between Wash 2 and 3. There was a significant ($p<0.0001$) increase between Wash 3 and the simulated transfusion.

Compared to Pre-storage samples, there was a significant ($p<0.0001$) decrease in platelets after storage and each wash (Wash 1, $p<0.0001$; Wash 2, $p<0.0001$; Wash 3, $p=0.0003$). However, when compared to Post-storage samples, there was no difference in platelet count following each wash, but there was a significant ($p=0.0006$) increase between Wash 3 and the simulated transfusion.

The erythrocyte morphology results are presented in Table 2.3.

Erythrocyte osmotic fragility

The MCF results are summarized in Table 2.2. The was a significant ($p=0.0369$) decrease in the MCF between Pre-storage and Post-storage. Compared to the Post-storage sample, there was a significant increase in MCF following Wash 1 ($p=0.0003$), Wash 2 ($p=0.0012$), and Wash 3 ($p<0.0001$).

Eicosanoid analysis

The eicosanoid results are summarized in Table 2.4. AA concentrations significantly ($p=0.0006$) increased during storage, but compared to Pre- and Post-storage samples, arachidonic acid concentrations progressively decreased following each wash. Additionally, compared to Post-storage samples, the AA concentration had significantly ($p<0.0001$) decreased following the simulated transfusion. Compared to Pre-storage, the TXB_2 concentrations decreased significantly ($p=0.0417$) after the first wash, but no other subsequent washes. Compared to Post-storage

samples, the TXB_2 concentrations increased (p=0.0337) after the simulated transfusion. There were no changes in HODE concentrations during storage. Compared to Post-storage levels, there was a decreased in HODE concentration after the third wash and simulated transfusion (p=0.0136 and p=0.0002, respectively). HETE concentrations increased significantly (p=0.0003) during storage and did not decrease to below Pre-storage levels until Wash 3 (p=0.0178).

Aerobic and anaerobic cultures

For the Post-storage samples, three units had a faint growth of a *Bacillus sp*. on enrichment broth. There was no aerobic bacterial growth identified on the Post-transfusion samples. Anaerobic bacterial growth was not detected in any sample.

Discussion

The manual washing technique used in our study reduced several plasma storage lesions that had developed in units of stored canine whole blood. However, three sequential washes adversely affected erythrocyte health by causing an increase in erythrocyte fragility, RDW, MCV, hemoglobin concentration, and changes in erythrocyte morphology. Fortunately, most of the measured plasma storage lesions decreased to below pre-storage levels following a single wash, suggesting that only one wash would be needed to effectively remove most of the storage lesions that had accumulated in stored units of blood.

Similar to previous studies, our study demonstrated an increase in lactate and pCO_2 concentrations during storage[14,28-30]. During storage, erythrocyte metabolic function continues, but in an anaerobic environment, requiring the conversion of intra-cellular pyruvate to lactic

acid[31]. As previously demonstrated, in both canine and human blood products, lactate begins to increase within 7 days of storage[14,28]. The excess lactate promotes the conjugation of bicarbonate to pCO_2 via carbonic anhydrase, and both lactate and pCO2 can contribute to the decrease in the pH of stored units, which was also demonstrated in our study. Following the first wash, our study demonstrated a significant decrease in both lactate and pCO_2, with the pCO_2 falling below pre-storage levels. However, despite this decrease in lactate and pCO_2 levels, the pH inside the unit continued to decrease, even after 2 additional washes. A potential explanation for the progressive decrease in pH is continued anaerobic metabolism. During washing and simulated transfusion, the erythrocytes remain in a low oxygen state and still require anaerobic metabolism. Although the lactate concentrations in the unit decreased after the first wash, these levels did not return to pre-storage levels, suggesting continued erythrocyte production of lactate. A second potential explanation for the progressive decrease in pH is the loss of the buffer, TCO_2, during the washing process. A third potential explanation for the continual decrease in unit pH, is the wash solution used in our study. The solution used in our washing technique, 0.9% NaCl, has a pH of 5.0, and it is unknown if the use of a more pH-neutral wash solution would have prevented the pH of the blood product from continuing to decrease.

Similar to previous studies, our study demonstrated an increase in potassium levels during storage[14,17,20,28,30,31]. The increase in potassium could be due to ATP depletion within the unit, and to the inhibitory effects that low temperatures have on the enzymatic function of Na-K-ATPase activity[33]. Makhro et al. demonstrated an increase in RBC sodium uptake and a loss of intracellular potassium from red cells during storage and shipment[33]. An increase in plasma potassium levels during storage has been associated with significant complications, including *in vitro* hemolysis during storage[28,34,35].

The transfusion of blood products with increased levels of potassium has not been associated with clinically significant adverse events[28]. However, serum potassium concentration could potentially increase during massive transfusions[36], especially in patients that are small and have concurrent hyperkalemia.

In both human and veterinary medicine, storage of blood products can increase erythrocyte fragility, causing an increase in *in vitro* hemolysis and resultant free hemoglobin concentrations[15,29,35,38,39]. The transfusion of hemolyzed blood product can lead to life-threatening acute hemolytic transfusion reactions[1]. An increase in red cell fragility and hemolysis can result in the release of cell free hemoglobin, which can contribute to the promotion of multiple organ failure[40], lung injury[41-45], nosocomial bacterial infections[19], systemic and pulmonary hypertension[41], kidney injury and endothelial dysfunction[21]. Hemoglobin can effectively scavenge nitric oxide, causing endothelial dysfunction, vasoconstriction, platelet aggregation, increased vascular permeability, and leukocyte adhesion[46]. In the present study, while there was no change in free hemoglobin during storage, free hemoglobin then increased after the first, but not subsequent washes, suggesting that washing stored blood products could increase the risk of *in vitro* hemolysis. A similar finding was demonstrated with a cell salvage device that also used 0.9% NaCl to wash canine erythrocytes[47]. Therefore, careful handling of these units, including using the least number of washes, would reduce risk of *in vitro* hemolysis. Our study also showed an increase in free hemoglobin following the simulated transfusion when compared to the final wash. One potential explanation for this increase in hemoglobin is continued decline in RBC health while held at room temperature, instead of refrigeration[33].

Cortes-Puch et al evaluated the effects of washing old versus fresh blood prior to transfusion in a canine pneumonia model and found that washing older blood reduced multiple

organ injury, lung injury, plasma iron and cell free hemoglobin levels, and improved survival times[42]. However, washing of fresh blood caused RBC membrane damage and increased cell free hemoglobin and iron, and worsened infection and outcomes[42]. A second study found that even with different transfused volumes of stored blood, plasma iron and cell free hemoglobin increased and worsened lung injury and mortality rates. Although washing of blood stored for 14-35 days did result in a separation of Kaplan Meyer curves, the differences in survival in washed vs non-washed blood stored for that length of time were not statistically different[43]. Further studies into the *in vitro* effects of washing units of stored blood prior to transfusion are needed in veterinary medicine.

Studies in both human and veterinary medicine have demonstrated that the progressive depletion of ATP during storage can cause increased senescent signaling through the expression of the negatively charged cell phospholipid, phosphatidylserine[37,48] and degenerative changes to erythrocyte shape, including echinocytes and spherocytes[39,49,50]. Our study showed that echinocytes were detected in 7 dogs in the Pre-storage, Post-storage, and Wash 1 groups, but that only one dog demonstrated echinocytes following the second wash. This may be the result of RBC lysis and removal of these fragile cells throughout the washing process. Rouleax and loss of central pallor were other red cell changes seen with storage. One possible cause for the increase in rouleaux formation is an increase in beta or gamma globulins, that are produced by activated leukocytes in the unit. Finally, the loss of central pallor, increased RDW and MCV, and decreased MCHC with washing supports previous reports of human and canine red blood cell swelling after manual or automated RBC washing[47,51]. The cellular swelling during washing is widely considered to be secondary to build up of osmotically active substances (such as lactate, chloride, and 2,3-DPG) intracellularly[49,52]. These osmotically active substances develop in RBCs

during storage and, after washing, the extracellular osmolality decreases significantly depending on the washing media used[53,54], resulting in an intracellular shift of fluid. Additionally, a storage-related dysfunction of Na-K-ATPase that may cause an increase concentration of intracellular sodium may also lead to an osmotic pull of water into the RBC, and cell swelling[33,39,47]. Increased MCV due to erythrocyte swelling shortens the lifespan of erythrocytes and increases osmotic fragility due to the decreased surface area to volume ratio and decreased deformability[47,52]. In human, washing with solutions containing dextrose, mannitol, or albumin that exhibit osmotic or colloidal oncotic effects improve red cell morphology as evidenced by normalized MCV and increased MCHC, as well as decreased erythrocyte water content and improved cell health[23,24,51,54]. Future studies in washing stored canine red cells are needed to determine optimal washing media to mitigate these changes seen to red cells.

Ionized calcium and ionized magnesium increased following each wash, suggesting that any residual and unbound CPDA was removed during the washing process. The use of washed blood products may provide a clinical benefit for patients with a concurrent coagulopathy or receiving massive transfusions, especially with hepatic dysfunction. Citrate toxicity is an uncommon but potentially harmful transfusion complication. Any unbound and excess citrate from the CDPA is usually quickly metabolized and eliminated from the patient. However, during massive transfusions, especially in patients with hepatic dysfunction, citrate metabolism can be reduced, causing *in vivo* calcium chelation and clinical hypocalcemia. Although our study did not measure citrate concentration in the unit, increases in both calcium and magnesium after the first wash suggest removal of most citrate. In fact, there was a progressive increase in iCa and iMg following each wash. With each wash step, it is possible that intracellular, calcium and magnesium leaked out of the RBCs, which could indicate cellular fragility and damage during

the washing process. Another possible cause for the increased ionized calcium is platelet activation; however, citrated storage media has been shown to decreased both platelet and leukocyte activation and, after prolonged storage, this mechanism is not expected to play a large role in the change in calcium levels measured in this study[55]. Finally, approximately 40% of calcium is bound to albumin. During the washing process, albumin was removed along with the plasma after the first washing step and so it is possible that the ionized calcium levels seen in this study were moved into the washing media to maintain calcium homeostasis.

Eicosanoids such as $PGF_{2\alpha}$, PGE_2, PGD_2, TXB_2, 6-keto-$PGF_{1\alpha}$, HETEs, and HODEs are signaling molecules that are derived from AA and are involved in multiple physiologic processes, such as vascular and bronchial tone, platelet activation, and renal blood flow[56]. Elevated levels of eicosanoids have been suggested as a possible cause of TRALI in humans[57], and a reduction in eicosanoids has been proposed as a mechanism to reduce transfusion reactions[3,58]. In humans, the use of leukoreduction filters prior to storage can reduce the accumulation of eicosanoids in stored units[5]; however, in dogs, leukoreduction causes an immediate increase in TXB_2 and $PGF_{2\alpha}$, and does not decrease 6-keto-$PGF_{1\alpha}$[8]. Our study demonstrated that manual washing decreased TXB_2 and PGE_2 levels, following one and two washes respectively, when compared to pre-storage levels. These results are similar to studies in units of human blood, which showed that washing caused a 95% reduction of eicosanoids that accumulated during storage, including TXB2 and PGE_2[3].

HETEs and HODEs are lipoxygenase (LOX) induced oxidative byproducts of AA that have vasoactive effects. 12-HETE directly contributes to platelet activation and aggregation, and influences the expression of the platelet adhesion molecule P-selectin[59]. HODEs are proinflammatory eicosanoids that contribute to leukocyte migration; specifically, human and

bovine neutrophil chemotaxis[60] and cytotoxic NK cell migration[61]. Increases in both of these eicosanoids, and HETEs in particular, have recently been associated with vascular dysfunction, hypertension, and microalbuminuria in greyhounds[62]. Our study is the first to demonstrate the presence of 12-HETE and HODEs in stored canine whole blood; however, the roles that 12-HETE and HODEs play in transfusion reactions are unknown, and further studies are needed. Eicosanoids have the potential to cause profound vascular effects in recipients, such as vasoconstriction mediated by thromboxane and HETEs[62,63]. In mice, the accumulation of HETEs and HODEs during storage is associated with a poor 24-hour RBC recovery in mice post-transfusion[64]. Furthermore, AA and HETEs have been implicated as the "second hit" in TRALI in people[57]. Other potential clinical consequences of increased exposure to eicosanoids include immunomodulation[65], enhancement of the systemic inflammatory response syndrome (SIRS)[66-69], and leukocyte and platelet activation[59,70,71]. Further investigation into the effects of eicosanoids and their physiologic effects and clinical significance in transfusion recipients is needed.

The present study had several limitations. Firstly, there were numerous technical problems with the blood gas analyzer that caused absent or partial data for several analytes. Secondly, one blood unit ruptured during the final centrifugation, which prevented the collection of the post-transfusion samples. Thirdly, our study only evaluated one type of wash media, 0.9% NaCl, and the use of different wash solutions, such as solutions that are buffered and physiologically similar to plasma, could have caused less cellular damage and potentially reduced the amount of hemolysis[24,72]. We chose 0.9% saline as the wash solution for use in this study because it has previously been used in humans to reduce storage lesions, and is readily available in most veterinary clinics[10,23,24]. Similarly, this study only used one washing protocol,

centrifugation speed and time, and other protocols have been reported in human transfusion medicine[10,23,24]. In dogs, additional studies are needed to evaluate which wash solution and centrifugation protocol best eliminates storage lesions and maintains cell health.

The present study demonstrates the manual washing of units of canine whole blood significantly reduced most storage lesions within the units, with many lesions attaining well below pre-storage levels. Based on the results of this study, we suspect that a single wash is all that is required to eliminate most storage lesions. Further investigation into alternative washing solutions and protocols may enable greater reductions in plasma storage lesions with minimal effects on erythrocyte health.

Table 2.1 Blood gas analysis

	Pre-storage*	Post-storage	Wash 1†	Wash 2‡	Wash 3	Transfusion*†‡
pH	6.91 ± 0.16	6.63 ± 0.04^{a}	6.59 ± 0.08^{a}	6.44 ± 0.31^{b}	6.20 ± 0.43^{bc}	5.88 ± 0.31^{c}
pCO_2 (mmHg)	53.1 ± 12	100.7 ± 16.1	35.8 ± 6.7	14.5 ± 6.8	4.7 ± 3.4^{a}	3.2 ± 4.1^{a}
pO_2 (mmHg)	89 ± 10^{a}	95.7 ± 36^{a}	107.4 ± 45.6^{ab}	105.8 ± 34.8^{b}	106.3 ± 28.1^{bc}	112.4 ± 42.3^{c}
sO_2 (%)	91.3 ± 5.5^{a}	84.1 ± 7.5^{b}	87.9 ± 6.3^{ab}	88 ± 5.9^{ab}	89.3 ± 5.3^{ac}	89.7 ± 5.3^{bc}
TCO_2 (mmol/L)	12.4 ± 2.2^{a}	13.8 ± 1.7^{a}	3.7 ± 1.5	2.5 ± 0^{b}	2.5 ± 0^{bc}	2.5 ± 0^{c}
MetHb (%)	0.6 ± 0.2^{a}	1 ± 0.4^{a}	1.9 ± 0.4^{b}	1.8 ± 0.4^{b}	2 ± 0.4^{b}	1.5 ± 0.3
Tbili (mg/dL)	1.8 ± 0.4^{a}	2.5 ± 1.8^{ab}	3.1 ± 2.1^{c}	3.1 ± 1.8^{c}	3.2 ± 2^{c}	1 ± 0.6^{b}

Blood gas analysis results (mean ± standard deviation) from 8 units of canine whole blood at donation (Pre-storage), storage (Post-storage), three washes with 0.9% NaCl (Wash 1, Wash 2, Wash 3), and after a simulated transfusion (Transfusion).
Comparison was not performed between samples that share the same symbols *, ǂ, and ǂ. Results that do not share a letter differed significantly (P <.05) on posthoc analysis.

Table 2.2 CBC results

	Pre-storage*	Post-storage	Wash 1†	Wash 2‡	Wash 3	Transfusion*†‡
WBC 10^3/ul	4.43 ±1.25[a]	4.69 ±1.35[abc]	3.86 ±1.41	3.4 ±1.46[d]	3.22 ±1.47[bd]	5.22 ±1.55 [c]
RBC 10^6/ul	5.06 ±0.82[a]	5.47 ±0.82[ab]	5.36 ±0.82[a]	5.17 ±0.82[a]	5.04 ±0.82[a]	7.01 ±0.87[b]
HGB g/dl	12.51 ±2.00[a]	13.28 ±1.99[abc]	13.25±2.00[b]	12.76 ±1.99[ab]	12.58 ±2.00[ab]	17.54 ±2.14[c]
HCT %	34.61 ±6.64[a]	37.76 ±6.64[ab]	39.83 ±6.62[b]	38.43 ±6.61[b]	37.46 ±6.61[ab]	53.4 ±7.10
MCV fL	68.5 ±4.51[a]	69.08 ±4.37[a]	74.45 ±4.22 [b]	74.54 ±4.19[b]	74.58 ±4.15[bc]	76.41 ±4.13[c]
MCH pg	24.78 ±0.88[ab]	24.28 ±0.72[a]	24.79 ±0.69[ac]	24.78 ±0.73[ac]	25.03 ±0.75 [bcd]	24.99 ±0.75[d]
MCHC g/dl	36.18 ±1.92[a]	35.15 ±1.91[a]	33.35 ±1.89[b]	33.3 ±1.89[b]	33.61 ±1.90[bc]	32.74 ±1.90[c]
RDW	14.64 ±1.14[a]	15.28 ±1.13[ab]	15.85 ±1.13[bc]	16.03 ±1.14[c]	15.95 ±1.14[c]	17.1 ±1.19
PLT 10^3/ul	149 ±38.82	91.25 ±38.76 [ab]	73.63 ±39.28 [a]	81.63 ±39.15 [ac]	84.75 ±39.11 [bc]	146 ±39.62
MCF (%)	0.487 ±0.091[a]	0.430 ±0.091[b]	0.504 ±0.084[ac]	0.490 ±0.083[acd]	0.575 ±0.080[cde]	0.569 ±0.080[e]

Mean ± deviation CBC parameters and MCF results in 8 units of canine whole blood from healthy donors at the time of donation (Pre-storage), after 28 days of storage at 4°C (Post-storage), after 1 wash with 250ml 0.9%NaCl (Wash 1), after the second wash with 250ml with 0.9%NaCl (Wash 2), after the third wash with 0.9% NaCl (Wash 3), and after reconstitution with 100ml 0.9%NaCl and a simulated transfusion over 5 hours (Transfusion).
Comparison was not performed between samples that share the same symbols *, ł, and ł. Results that do not share a letter differed significantly (P <.05) on posthoc analysis.

Table 2.3 Erythrocyte morphology results

Morphology	Pre-storage	Post-storage	Wash 1	Wash 2	Wash 3	Transfusion
Echinocytes	7 (rare to moderate)	7 (moderate to many)	7 (moderate to many)	1 (moderate)	ND	ND
Codocytes	1 (rare)	3 (rare)	ND	ND	ND	ND
Keratocytes	5 (rare to mild)	1 (rare)	ND	ND	ND	ND
Eccentrocyte	1 (rare)	ND	ND	ND	ND	ND
Dacrocyte	ND	ND	2 (mild to moderate)	2 (rare to mild)	2 (rare to mild)	2 (rare to mild)
Ovalocyte	ND	ND	1 (mild)	3 (rare to mild)	1 (rare)	2 (rare to mild)
Poikilocytosis	1 (mild)	1 (mild)	ND	ND	ND	ND
Other Descriptions						
Smudge cells	ND	ND	8 (mild to many)	7 (many)	7 (many)	7 (many)
Rouleaux	ND	ND	3	5	7	3
Lack of Central Pallor	ND	ND	5	8	8	7
Cells Not Spread Out	ND	ND	3	3	3	4

Abnormal erythrocyte morphology for samples at the time of unit of canine whole blood collection (Pre-storage), after 28 days storage at 4°C (Post-storage), after the first (Wash 1), second (Wash 2), and third (Wash 3) wash with 250ml of cold 0.9% NaCl, and after a 5 hour simulated transfusion at 25°C (Transfusion).
Results reported are the number of samples for which the RBC and WBC morphology was present. Frequency of abnormalities was scored by use of a subjective scale as rare (1-10 abnormal cells/hpf), mild (11-50 abnormal cells/hpf), moderate (51-100 abnormal cells/hpf), and many (>100 abnormal cells/hpf). ND= not detected.

Table 2.4 Eicosanoid results

	Pre-storage*	Post-storage	Wash 1†	Wash 2‡	Wash 3	Transfusion*†‡
AA (pmol/ml)	56.0 ±42.2	96.8 ±41.5	18.3 ±40.0	3.7 ±40.1	0.9 ±40.2	0.3 ±40.2
$PGF_{2\alpha}$ (pmol/ml)	2.4 ±2.8	2.1 ±2.8	2.0 ±2.7	2.3 ±2.7	1.7 ±2.7	1.7 ±2.6
TXB_2 (pmol/ml)	7.2 ±49.6 [a]	6.7 ±49.6 [ab]	4.6 ±49.6 [b]	7.1 ±49.6 [ab]	9.0 ±49.6 [abc]	73.5 ±49.6 [c]
PGE_2 (pmol/ml)	0.4 ±0.3 [a]	0.2 ±0.3 [abc]	0 ±0.3 [ab]	0 ±0.3 [b]	0.1 ±0.3 [abc]	0 ±0.3 [c]
PGD_2 (pmol/ml)	0.082 ±0.1	0.059 ±0.1	0.004 ±0.1	0.004 ±0.1	0.069 ±0.1	0.009 ±0.1
6-keto-$PGF_{1\alpha}$ (pmol/ml)	0.004 ±0	0.005 ±0	0.005 ±0	0.000 ±0	0.001 ±0	0.008 ±0
12-HETE (pmol/ml)	34.5 ±63.7 [a]	129.4 ±65.5 [b]	106.9 ±68.9 [ab]	42.1 ±68.7 [a]	12.0 ±68.8 [c]	5.1 ±69.0 [c]
HODE (pmol/ml)	8.5 ±10.4 [ac]	11.1 ±10.4 [ab]	8.0 ±10.2 [ac]	12.7 ±10.1 [ac]	4.8 ±5.4 [bc]	1.3 ±5.0 [b]

Mean ± deviation eicosanoid concentrations in 8 units of canine whole blood from healthy donors at the time of donation (Pre-storage), after 28 days of storage at 4°C (Post-storage), after 1 wash with 250ml 0.9%NaCl (Wash 1), after the second wash with 250ml with 0.9%NaCl (Wash 2), after the third wash with 0.9% NaCl (Wash 3), and after reconstitution with 100ml 0.9%NaCl and a simulated transfusion over 5 hours (Transfusion).
Comparison was not performed between samples that share the same symbols *, ǂ, and ǂ. The shaded results could not be modeled during statistical analysis and therefore comparisons could not be performed. Results that do not share a letter differed significantly (P <.05) on posthoc analysis.

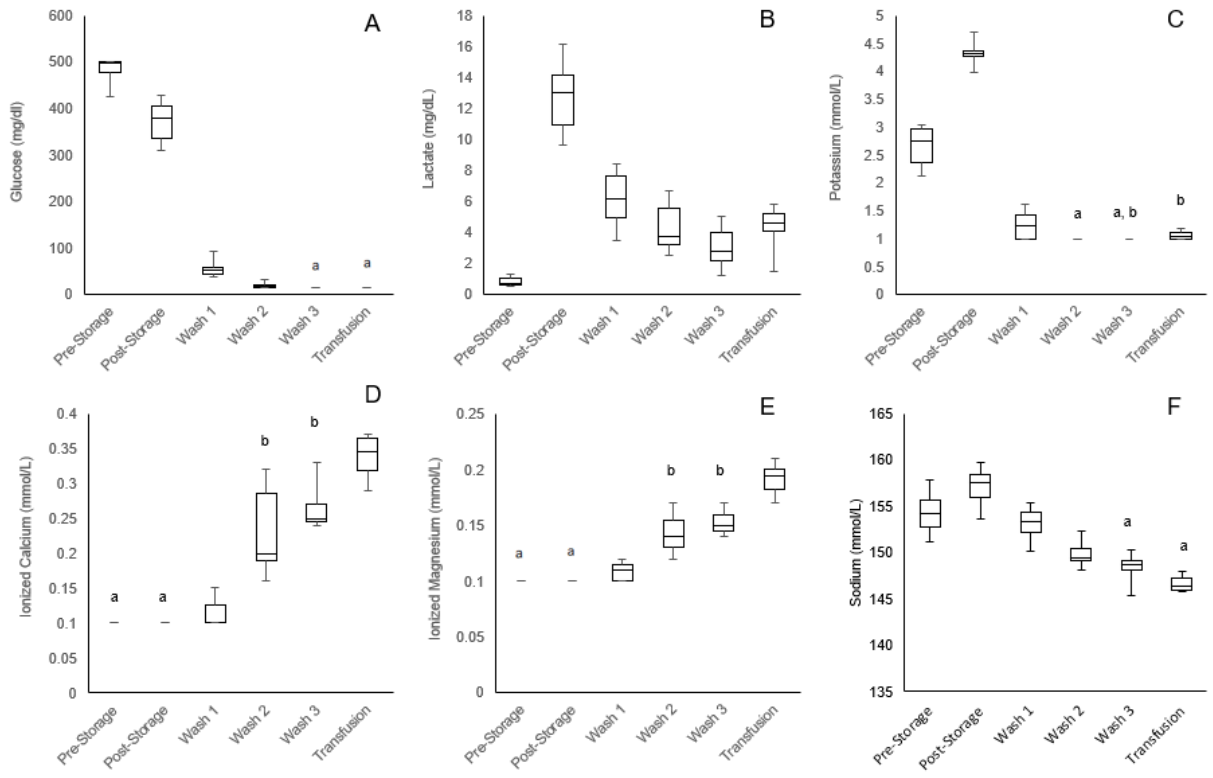

Figure 2.1 Select blood gas analysis results.

Blood gas analysis results (glucose (A), lactate (B), potassium (C), ionized calcium (D), ionized magnesium (E), sodium (F)). The box and whiskers plot demonstrate the median (line), interquartile range (box), and total range (whiskers). Results that do not share a letter differed significantly (P <.05) on posthoc analysis. Pages 40-41.

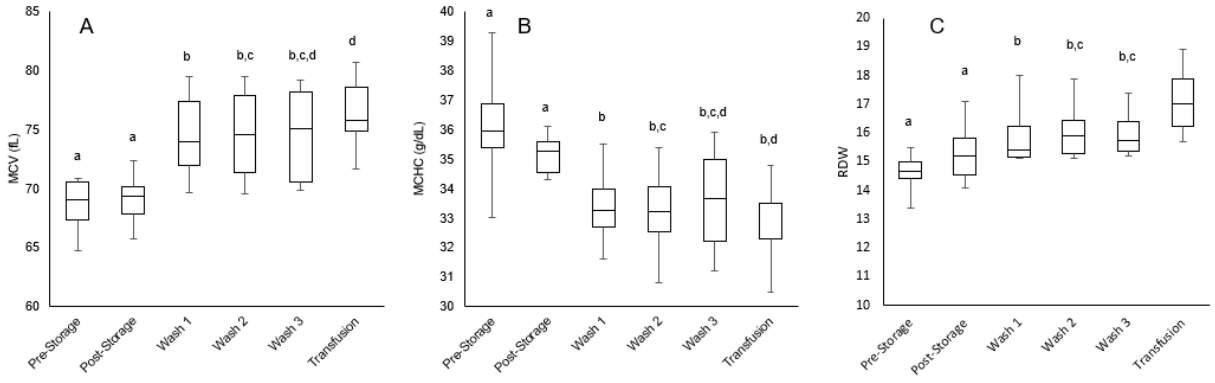

Figure 2.2 Select CBC results

CBC results (MCV (A), MCHC (B), RDW (C)). The box and whiskers plot demonstrate the median (line), interquartile range (box), and total range (whiskers). Results that do not share a letter differed significantly (P <.05) on posthoc analysis. Pages 41-42.

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CHAPTER III

CONCLUSION

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The work provided in this thesis details a manual washing technique, previously used in human transfusion medicine[1], utilized to wash stored canine red blood cells. The technique was simple to perform with minimal time, skill, and supplies needed. Furthermore, our research showed that manual washing of storage canine units of red blood cells successfully reduced concentrations of plasma storage lesions.

As this thesis has demonstrated that washing units of canine red cells can effectively reduce plasma storage lesions, future studies should focus on improving cell health through altering the technique. The first washing step appeared to be the most successful in reducing storage lesion concentration. Therefore, recommendations for future use may likely consist of a single washing step. As subsequent washes occurred there were some unwanted consequences of the washing procedure, including red cell swelling. In humans, various washing media have been explored to try and improve cellular health, with options including PlasmaLyte®, 1 and 5% albumin products, deglycerolization media, and mannitol-dextrose solutions[2-5]. While no extensive studies exist to allow comparison of all washing media available, there were several

reports that solutions with colloidal components results in less hemolysis compared to others[1-3,5,]. Future studies may include incorporating different washing media into the design to determine the best media to maintain cell health. In addition to washing media, future studies should also investigate the optimal centrifugation speed and time, as there are several manual techniques described in human literature with no single effective technique described[2,4,6].

Although these studies may show improved cell health through altering the washing technique, the manual washing technique described here effectively reduced plasma storage lesions within units of stored canine red blood cells. A washing technique may benefit patients receiving massive transfusions (>90 ml/kg in 24 hours or >45 ml/kg in 3 hours)[7]. Dogs receiving massive transfusions have been well documented to develop citrate toxicity, in which the excessive amounts of citrated anticoagulant chelate the patient's calcium in vivo[7]. The resulting hypocalcemia can produce significant clinical signs in patients undergoing massive transfusions and can even be life-threatening[7]. Taking the short time to wash the units of stored blood prior to transfusing stored blood to these patients can reduce this risk almost completely based on this study's results.

Another patient group that may benefit from the process of washing red cells prior to transfusion are patients with known severe infections requiring transfusion, when only older (>14 days of storage) stored blood is available[8-14]. Previous studies have demonstrated the negative consequences of excessive iron availability, in the form of cell free hemoglobin, to bacteria in sick patients[8-14]. Given that excess cell free hemoglobin aids in bacterial growth, washing older stored units of blood prior to transfusion in patients with severe bacterial infections can help prevent worsening outcome and decrease mortality rates[8-14].

A theorized situation in which washing stored units of blood prior to transfusion may be beneficial includes transfusion of diabetic patients. To this author's knowledge, there are no published studies investigating post-transfusion blood glucose levels of diabetic dogs. This study demonstrated that, post-storage, there remained high concentrations of glucose within the unit of blood. Once transfused, that extra glucose has the potential to cause post-transfusion hyperglycemia in diabetic patients, due to their lack of endogenous insulin. In poorly regulated diabetics with polyuria, polydipsia, electrolyte abnormalities, and increased risk of infections, washing stored blood prior to transfusion may significantly reduce the amount of excess glucose transfused into these patients, and may be of clinical benefit.

Other patients described in human medicine that may benefit from washing of stored red blood cells prior to transfusion include trauma patients (to reduce the risk of multiple organ failure[15]), pediatric patients undergoing cardiac bypass[16-18], pediatric patients requiring multiple transfusions[19], and humans with IgA deficiencies[20]. Research into the translation of these conditions into veterinary patients in general, and dogs in particular, is lacking. However, since older stored canine blood has been implicated in the etiology of hemolytic transfusion reactions and non-hemolytic transfusion reactions[21,22], washing cells prior to transfusion may be a method of reducing these transfusion reactions, and so further research is indicated.

Washing of stored canine red cells prior to transfusion can provide several therapeutic benefits but, as demonstrated by Solomon et al., not all patients require washing of stored blood[23]. In some patients, like those experiencing hemorrhagic shock, transfusion of older non-washed blood reduced mortality rates and improved vascular function compared to younger blood[23]. Future studies are needed to explore the clinical impact of washing stored blood prior to transfusing in patients experiencing various illnesses.

A focus of newer studies in transfusion medicine is exploration of transfusion-associated immunomodulation[24]. The mechanisms of immunomodulation associated with transfusions is not well characterized, but storage lesions including cytokines, persistent leukocytes and platelets, and neutrophil extracellular traps are thought to play key roles[24-27]. While not a focus of this study, leukoreduction has been the primary method of reducing immunomodulatory effects of transfused stored blood[28-32]. It is possible that washing stored blood may also aid in reducing some of these immunomodulatory effects. Moreover, the combined effects of leukoreduction and cell washing have not been explored in canine transfusion medicine and, together, they may even more effectively reduce storage lesions and potential immunomodulation. Further research in this area is needed.

The study described in this thesis successfully demonstrated the reduction of storage lesions in canine red cell units through the use of a manual washing technique. The washing of stored blood prior to transfusion may have many as yet unexplored benefits, and future research opportunities in this area are boundless.

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