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Effects of Leukoreduction and Storage on Erythrocyte Phosphatidylserine Expression and Eicosanoid Concentrations in Units of Canine Packed Red Blood Cells

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Effects of leukoreduction and storage on erythrocyte phosphatidylserine expression and eicosanoid concentrations in units of canine packed red blood cells.

By

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Storage of canine packed red blood cells (pRBCs) can increase erythrocyte phosphatidylserine (PS) expression and eicosanoid concentrations. The objective of this study was to determine the effects of leukoreduction on erythrocyte PS expression and eicosanoid concentrations in stored units of canine pRBCs. Units of whole blood were leukoreduced (LR) or non-leukoreduced (non-LR), and stored (10 and 21 days) as pRBCs. Samples were collected at donation, and before and after a simulated transfusion. PS expression was measured by flow cytometry, and concentrations of arachidonic acid (AA), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), thromboxane B_2 (TXB_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and leukotriene B_4 (LTB_4) were quantified by liquid chromatography-mass spectrometry. Our study demonstrated that PS expression on the surface of erythrocytes was not affected by leukoreduction or storage duration. Additionally, the passage of blood through a leukoreduction filter causes an immediate and dramatic increase in TXB_2 and $PGF_{2\alpha}$ concentrations, but these concentrations then decrease during subsequent storage.

Despite leukoreduction, the concentration of 6-keto-$PGF_{1\alpha}$ continued to increase during storage and simulated transfusion. Overall, when compared to non-LR units, the addition of a leukoreduction step prior to storage had a minimal impact on the accumulation of eicosanoids in canine units of pRBCs. While leukoreduction may be beneficial in other aspects of transfusion medicine, based on the results in this study, using leukoreduction to decrease PS expression and eicosanoid concentrations does not appear to be effective.

DEDICATION

I dedicate this research to my mother, who has loved and supported me in every way possible.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
15-PGDH	15-hydroxyprostaglandin dehydrogenase
AA	Arachidonic acid
COX	Cyclooxygenase
LOX	Lipoxygenase
LR	Leukoreduce
LTB_4	Leukotriene B_4
MFI	Median Fluorescence Intensity
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFS	Prostaglandin F synthase
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
pRBCs	Packed Red Blood Cells
PS	Phosphatidylserine
TXB_2	Thromboxane B_2

CHAPTER I

INTRODUCTION

History of Transfusion Medicine

Blood transfusions are commonly used for the treatment of life-threatening anemia in today's critical veterinary patients. Modern day blood transfusion techniques were developed not in a single day's achievement, but rather through a long journey and contribution from many researchers. In humans, blood transfusions have been used as life saving techniques for many, many years. In contrast, although the first blood transfusion recorded was performed in a dog, veterinary transfusion medicine has somewhat lagged, only significantly developing over the past several decades.

The first documented transfusion in any species occurred in 1665 when Richard Lower withdrew blood from one dog and replaced it with blood from another dog. Over the next several decades, transfusing of blood from animals to humans was described but quickly outlawed due to the high rate of fatal reactions.[1,2] It wasn't until 1818, 150 years after the first recorded canine transfusion, when James Blundell, a British obstetrician, performed the first successful transfusion of human blood to a patient for the treatment of postpartum hemorrhage. Using the patient's husband as a donor, he extracted a small amount of blood from the husband's arm and, using a syringe, he successfully transfused the wife. During the 19th and 20th centuries, human transfusion medicine exploded with the development of sterile technique by Joseph Lister, the discovery of human blood

groups A, B and O by Karl Landsteiner, the establishment of anticoagulants, blood banking systems and the American Red Cross as well as the development of stringent blood storage and transfusion standards regulated by the Food and Drug Administration.[2]

Although much blood transfusion research was originally performed with animals, veterinary transfusion medicine was not established until recently, most likely secondary to shifting views of animals as companions rather than property, and the desire for better animal health care. Due to the increasing availability of equipment and facilities over the past several decades, transfusion of various blood products to companion animals has become a common practice in not only advanced care facilities, but general practices as well. As knowledge, has grown and the availability of different blood products has increased, however, transfusion therapy has become more complex.

Blood from animal donors may be collected and immediately transfused, or collected and stored for use in patients at a later date. Furthermore, blood can be separated into different components, including but not limited to, packed red blood cells, platelet concentrate, plasma and cryoprecipitate.[3,4] Component products allow clinicians to tailor-make treatment plans most appropriate for the individual patient. To further increase the availability of blood products, storage of these products becomes necessary, and can result in significant changes within the unit. These changes are known as "storage lesions" and can contribute to reactions and decreased survival of the transfused product within the recipient.[5,6] Furthermore, several methods have been developed to avoid these complications including cell washing and leukoreduction.[6] This manuscript is

intended to describe multiple aspects of veterinary transfusion medicine including transfusion reactions, storage lesions and leukoreduction techniques.

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CHAPTER II

REVIEW OF THE LITERATURE

Transfusion Reactions

With the increasing availability of canine and feline blood products, transfusion reactions are frequently encountered. Consequently, significant strides have been made in the understanding of these reactions and how to limit their occurrence in our veterinary patients. The incidence of transfusion reactions may vary depending on several factors including storage time, storage techniques, underlying disease of the recipient, type of product transfused and amount of product transfused. Currently, transfusion reactions occur in 3-28% of canine and feline transfusions.[1–6]

Transfusion reactions can be categorized into acute and delayed, as well as immunologic and non-immunologic in origin. Acute immunologic reactions include febrile non-hemolytic reactions, acute hemolysis, allergic/anaphylactic reactions and transfusion-related acute lung injury (TRALI). The most common types of acute immunologic reactions include febrile non-hemolytic and allergic reactions however the most life-threatening reaction encountered is acute hemolysis.[7–10] Delayed immunologic reactions may occur days to months following a transfusion. Types of delayed immunologic reactions include hypersensitivity reactions II to IV which may manifest as serum sickness, thrombocytopenia and/or shortened red blood cell life span.[9,10]

Immunologic reactions result from interactions between the recipients immune system and a variety of cells, compounds and inflammatory mediators within the blood product. Antibody-antigen interactions, especially directed against leukocytes and platelets within blood products, can result in an increase in systemic levels of cytokines and other pro-inflammatory mediators. Antibody binding to transfused mast cells and basophils can lead to degranulation and secretion of pro-inflammatory and vasoactive substances such as histamine, cytokines, and eicosanoids. The ability of transfusions to modulate the recipient's immune system is well established and characterized by the term transfusion-related immunomodulation (TRIM).[7,11,12] Underlying physiologic mechanisms behind TRIM are multifactorial and determined not only by the immunogenic potential of the transfused product, but also by the recipients underlying disease process and genetic predisposition. The benefits of transfusion-induced immunomodulation was first described in renal transplant recipients in which immunosuppressive effects resulted in higher success rates.[13] Deleterious effects have since been described and are linked to manifestations such as viral activation, increased occurrence of postoperative infection and TRALI.[11,14] Described mechanisms by which allogeneic transfusions may cause immunomodulation include depression of cellular immunity by downregulation of T-helper type 1 and upregulation of T-helper type 2 cells, dysregulation of innate immunity during inflammation by decreased natural killer cell and macrophage function as well as neutrophil priming and endothelial cell activation.[11,15] The "two-insult" model of post-transfusion injury proposes that an initial insult such an underlying inflammatory

condition or trauma primes the patient's immune cells or endothelium, and further inflammation is triggered by a second immunomodulating insult (eg, transfusion), resulting in an unrestrained inflammatory response. This theory maybe the underlying mechanism behind TRALI.[14,16] Transfusion-related acute lung injury is the most common cause of transfusion-related deaths in human, however, despite the theoretical background for TRIM and TRALI, conclusive evidence of adverse clinical consequences of immunomodulation is lacking in veterinary patients and warrants further investigation.

Regarding non-immunologic transfusion reactions, the most common include infectious disease transmission, sepsis from bacterial contamination of the unit, citrate toxicity leading to hypocalcemia (in patients receiving massive transfusions), and circulatory overload.[9,17] Typically, clinical signs of a transfusion reaction manifest early in transfusion administration and may include fever, urticaria, facial edema, vomiting, weakness, tachycardia or bradycardia, tachypnea, dyspnea, hypotension, hypothermia and collapse.[5,18] Signs consistent with a transfusion reaction can often be masked by the clinical signs associated with the underlying immune mediated disease.[3,5,10,17,18]

With the expansion of veterinary transfusion medicine, standardized protocols regarding product processing and storage, donor and recipient testing, product administration and monitoring/treatment of reactions have been developed to best utilize blood products while avoid transfusion reactions.[19,20] Processing of veterinary blood products primarily consists of sterile collection via a blood collection set into a storage bag containing an anticoagulant preservative. Products may then be separated into

components (such as fresh plasma, packed red blood cells, platelets concentration and cryoprecipitate) or stored as whole blood. Each product may require a specific storage environment and storage time. For example, whole blood is stored in a refrigerator at 4°C for a maximum of 35 days whereas fresh plasma may be frozen at -40°C and stored for 1 year.[9] Proper storage of blood products decreases the risk of both immunologic and non-immunologic reactions.

Careful selection of donor animals should be performed. Perhaps the most important criterion for donors is to meet minimum standards for infectious disease testing. According to the most recent consensus statement regarding canine and feline blood donor screening for blood-borne pathogens, minimal standards for dogs should include a negative test result for *Anaplasma platys* and *phagocytophilum* PCR, *Ehrlichia canis* PCR or serology, *Babesia canis vogeli* and *Babesia gibsoni* PCR, *Bartonella henselae* and *vinsonii* subspecies *berkhoffi* PCR and *Mycoplasma haemocanis* PCR. Minimal standards for cats include negative testing for *Anaplasma phagocytophilum* PCR or serology, *Bartonella henselae* PCR, *Mycoplasma haemofelis* PCR, feline leukemia virus antigen testing and feline immunodeficiency virus antibody testing. Infectious agents for which testing is recommended are chosen based on the agents documented ability to cause clinical infection in recipients after blood transmission, ability of the agent to cause subclinical infection such that carriers might inadvertently be identified as healthy blood donors, whether or not the agent can be detected using culture or molecular methods from the blood of an infected animal, and finally, whether or not the resultant

infection in the recipient has the potential to cause life-threatening illness and be difficult to eliminate with antimicrobial drugs.[21]

Blood typing and cross matching are methods available to identify compatible blood products prior to administration, in an effort to avoid immunologic reactions. Canine blood types are characterized by the antigens present on the red blood cell. While many canine antigens have been identified by sensitizing dogs with canine allogeneic blood, only dog erythrocyte antigens (DEA) 1.1, 1.2, 3, 4, 5 and 7 can be routinely measured.[9] DEA 1.1 is highly antigenic and therefore can result in a severe hemolytic transfusion reaction if DEA 1.1 positive blood is administered to a sensitized DEA 1.1 negative dog. Screening dogs for the presence or absence of this antigen is recommended prior to transfusion.[8,9] DEA 1.1 typing is readily available as a point-of-care test and the Quick test DEA 1.1. The DEA 1.1 frequency of 42 to 71.2% varies with geographical location and breed.[8] Recently, another erythrocyte antigen, the Dal antigen has been identified on canine red blood cells and can be absent in the Dalmation and Doberman breed.[22] Furthermore, Kai 1 and Kai 2 are two recent blood types discovered by screening monoclonal antibodies. Discovery of Kai is the first discovery of canine blood types by use of this technique.[23] Feline blood types are determined by 3 alleles (A, ab and b) with A being dominant over the rare ab, which is dominant over b. Cats with a geno-type A/A, A/ab, or A/b are type A, whereas only cats with b/b are type B. Rare AB cats are genotypically ab/ab or ab/b. Type A occurs in the majority (97%) of cats in the United States while type B occurs in 0.3-6% of cats with a predilection for exotic breeds such as the Devon rex (41%), British shorthair (36%), Cornish rex (31%), exotic

shorthair (27%), and Scottish fold (19%). Type AB appears to be rare and occurs in approximately 1% of the cat population worldwide.[24] Recently, a feline blood antigen, termed Mik, has been identified and can play a role in blood incompatibility in cats that lack this antigen.[25]

Cross matching enables identification of recipient antibodies to donor red blood cells (major cross match) as well donor antibodies to recipient red blood cells (minor cross match). Point-of-care cross matching kits are available for rapid determination of blood compatibility. Current recommendations are that all donors and recipients should be blood typed and cross matched prior to transfusion.[8–10] In the canine species, an acute reaction is less likely to occur in a recipient receiving a transfusion for the first time. This is due to the lack of naturally-occurring alloantibodies against the majority of the DEA groups; specifically, DEA 1.1 alloantibodies have not been observed in the canine.[26] Therefore, on an emergency basis only, forgoing typing and cross matching is a viable option for dogs that have not received a prior transfusion or have not been otherwise sensitized. In the cat, typing and ideally cross matching should always be performed prior to a transfusion due to the presence of naturally-occurring alloantibodies and high risk of a severe transfusion reaction in this species if incompatible blood is administered.[8,9] For cats, point-of-care testing will identify A or B blood. In summary, with the majority of cases, pre-transfusion typing and cross matching should be performed in order to provide the most compatible product for the recipient to limit the risk of reaction and to limit antibody formation for future transfusions.

Despite screening donors, providing adequate processing/storage methods and identifying blood compatibility via typing and cross matching, transfusion reactions still occur and are likely related to the presence and development of substances within the blood product itself that can result in stimulation of the recipient's immune system. These immune-modulating substances include entire cells, cell particles, cytokines and lipid mediators. They can be present at the time of collection of the blood product, such as leukocytes and platelets, or may develop/accumulate during storage secondary to altered metabolism, oxidative stress and accelerated product degradation. The latter, termed storage lesions, are a subject of much research as they can be a significant contributor to product survival as well as immune responses in the recipient.

Storage Lesions

The occurrence of a transfusion reaction within the recipient is multifactorial however storage of blood products prior to administration has been associated with increased risk of reaction as well as morbidity and mortality in the recipient.[1,27,28] In an effort to ensure adequate preservation of the units and safety to the recipient, standards of canine and feline blood storage have been established and are largely based on human protocols in which red blood cell products are stored for no longer than 42 days following the Food and Drug Administration requirement that 75% of transfused red cells must remain in circulation 24 hours after transfusion and hemolysis must affect less than 1% of stored cells at the end of the storage period.[19,20,29] Regardless of these protocols, changes within the unit occur, and can be categorized into mechanical, biochemical and immunologic events.[12]

Mechanical events include alterations secondary to physical manipulation of the product including transient changes in ambient temperature, kinking of tubing, clogging of filters, centrifugation or rough handling that results in shearing or lysis of cells, damage to cellular membranes, decreased deformability of erythrocytes, increased phosphatidylserine expression and microparticle formation. Mechanical events may occur at any time, however most likely occur with increased incidence during handling such as in collection of the product, product filtration or transfusion to the recipient.

Biochemical events occur during storage, and result from the artificial environment in which the contents of the product are maintained. This artificial environment results in altered metabolism and oxidative stress. Biochemical changes include decreased adenosine triphosphate (ATP), 2,3 diphosphoglycerate (DPG) and nitric oxide (NO), as well as increased potassium leakage, ammonia accumulation and oxidative injury.

Because mammalian red blood cells do not contain mitochondria, they depend on anaerobic glycolysis to produce two ATP and two lactic acid molecules per one glucose molecule. An important enzyme in this pathway is phosphofructokinase which is inhibited by the progressingly acidic environment in storage. As a result, ATP production falls and results in membrane instability as well as failure of many intracellular processes such as phosphorylation of membrane phospholipids and proteins, membrane transport functions, synthesis of purine and pyrimidine nucleotides, and synthesis of glutathione.[12,30] Hemoglobin and 2,3 DPG bind resulting in reduced affinity of hemoglobin for oxygen in erythrocytes, and allowing effective release of oxygen to

peripheral tissues.[30] It is produced from a side pathway of anaerobic glycolysis and as a result of decreased metabolism, 2,3DPG decreases in storage. Importantly however, decreased 2,3 DPG has not been shown to affect oxygen delivery to the tissues when transfused to rats.[31] Nitric oxide is an uncharged radical produced by the vascular endothelium and is a major endogenous vasodilator. Within red blood cells, NO binds to a residue of hemoglobin to form S-nitrosohemoglobin (SNO-Hb). During storage, erythrocyte SNO-Hb bioactivity falls rapidly and is not restored following infusion into a recipient. It has been suggested that this may impair tissue oxygen delivery after RBC transfusions.[12,32] Oxidative reactions occur during normal metabolism and produce reactive oxygen and nitrogen species. Naturally, these products are reduced and result in minimal cellular injury. Mechanism to avoid oxidative injury depend largely on cellular glutathione levels.[30] In storage, decreased metabolism and ATP results in depleted glutathione and progressive cellular injury and destruction.[12,32] Through altered metabolism, oxidative stress and cellular injury, levels of free hemoglobin, potassium and ammonia increase in product supernatant.[12] Free hemoglobin secondary to hemolysis and hemoglobin-containing microparticles can react with NO after transfusion to form methemoglobin and nitrate.[33] The consequences of increased potassium and ammonia in the supernatant of veterinary blood products is of uncertain significance and additional research in this area is required.

Immunologic changes that occur in units during storage primarily result from interaction with the white blood cell population.[34] Leukocytes in packed red blood cell units can become activated, producing and releasing pro-inflammatory cytokines such as

interleukin-8.[35,36] Additionally leukocytes lyse early in storage and release enzymes that not only result in damage to the erythrocyte but also contribute a portion of pro-inflammatory and pro-thrombotic compounds including elastase, secretory phospholipase-A2, soluble FAS-ligand, prostaglandin E-2 and transforming growth factor-1.[37,38]

Phosphatidylserine

An increase in phosphatidylserine (PS) expression on the red cell surface is a well described red cell storage lesion of human blood products that results from damage/degradation of the erythrocyte.[39–41] Phosphatidylserine is a negatively charged phospholipid, comprising a portion of the erythrocyte cell membrane. The majority of cell membranes in the eukaryote, including the erythrocyte cell membrane, are comprised of a lipid bilayer with neutral phospholipids (phosphatidylcholine and sphingomyelin) on the outer leaflet of the cell membrane and negatively charged phospholipids (phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine) on the inner surface.[42]

In circulation, as the erythrocyte ages or becomes damaged, alterations occur to the cell membrane that allow for immune recognition and removal of the cell from circulation via phagocytosis. One change of interest is PS translocating to the outer cell membrane layer. At rest, PS is located within the inner leaflet of the plasma membrane, but with aged or damaged erythrocytes, PS expression will gradually translocate to the cell surface which will signal the need for cell removal via mononuclear phagocytic cells.[42] Consequently, in storage, the expression of PS on the surface of the red blood cell

serves as an indicator for erythrocyte health.[43] Other changes that occur in the aged/damaged erythrocyte includes an increase in senescent antigen formation, IgG binding, loss of cell adhesion molecules, vesicle formation and loss of cell pliability.[44,45]

On average, a canine erythrocyte's life span in circulation is 120 days, however, in storage, erythrocyte degradation occurs at a faster rate. Phosphatidylserine expression on the erythrocyte surface can be correlated to the availability of ATP. ATP allows function of the enzyme aminophospholipid translocase which actively flips PS back to the inner surface of the cells. Lack of ATP results in inadequate aminophospholipid translocase activity and increased PS expression.[46]

During storage, erythrocytes are primed for ATP depletion and intracellular potassium leakage which enhances PS expression on the erythrocyte surface.[39] Furthermore, increased PS expression in units of packed red cells may promote procoagulant activity. In other cell types, PS expression is involved in angiogenesis, thrombin formation, and the regulation of vascular tone and can serve as a marker for potential inflammatory and procoagulant activity.[40,41,47] Phosphatidylserine-expressing platelets in circulation play a pivotal role in activating the coagulation cascade.[48] Phosphatidylserine-expressing erythrocytes do not appear to cause a similar effect, possibly in part because senescent erythrocytes are cleared quickly from the circulation.[40] In stored blood products, however, the amount of PS expression can accumulate and potentially shorten the circulating survival time of the transfused erythrocyte, or contribute to transfusion-related complications. In human research, Lu *et al* demonstrated that increased erythrocyte PS expression in red blood cell concentrate is associated with

increased procoagulant activity, and that both the amount of PS expression and procoagulant activity increased with the length of storage.[41] Furthermore, leukoreduction (removal of white blood cells and platelets) of whole blood prior to storage can decrease this procoagulant activity.[40,49,50]

Several techniques have been developed to identify PS-expressing cells including the use of chemical reagents or phospholipases to alter the externalized phospholipids, techniques to monitor the redistribution of added phospholipid probe molecules across the cell membrane, and the activation of hemostatic processes such as PS-mediated activation of prothrombin to thrombin via prothrombinase.[51,52]

Flow cytometry is a technique available to identify and sort cell populations. Using flow cytometry, PS-expressing erythrocytes can be identified and quantified by using fluorescently labeled annexin V. Annexins are a family of proteins that bind to charged phospholipids particularly PS. Fluorescently labeled annexins have been used to identify PS on the surface of activated platelets under physiologic conditions in vitro as well as on the surface of vesicles released from activated platelets and apoptotic cells.[51,53] Radioactively labeled annexin V has been used to identify PS exposure in red cells.[53] However, binding of radiolabeled annexin V only indicates the loss of phospholipid asymmetry in the average cell and does not allow analysis of the erythrocyte population. Kuypers *et al.* described the technique in human red blood cells showing that fluorescently labeled annexin V used in conjunction with flow cytometry and fluorescent microscopy offers an excellent method for the determination of loss of normal phospholipid asymmetry in subpopulations or individual red cells.[46]

Eicosanoids

Eicosanoids are signaling molecules primarily derived from arachidonic acid. Eicosanoids include the prostaglandins, leukotrienes and lipoxins and are involved in a wide range of physiologic processes including the regulation of inflammation, vascular and bronchial tone, platelet aggregation, uterine and gastrointestinal motility, and renal blood flow as well as hypothalamic/pituitary hormone secretion.[54] Eicosanoids play an important role in modifying physiological processes in both homeostatic and inflammatory condition via their interaction with signaling molecules such as cytokines and chemokines.[54] Furthermore, eicosanoids can modify inflammatory responses depending on the type and location of the receptor to which they bind. Specifically, thromboxane promotes platelet aggregation, vasoconstriction and bronchoconstriction.[55] Thromboxane can also enhance the production of interleukin-8, a pro-inflammatory cytokine and potent neutrophil chemoattractant[55,56] Cys leukotrienes, those containing a cysteine residue, specifically leukotriene B_4, recruit neutrophils and other cells of inflammation to site of tissue damage. Other leukotrienes (specifically, leukotriene D4) trigger contractions in the smooth muscles lining the bronchioles and act in asthma and allergic disease. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) can regulate the cytokine response of mast cells, while PGI_2 is known to modulate immune cell functions, specifically T helper cells.[57,58] Lipoxins, which were the first eicosanoids to be identified and recognized as potential anti-inflammatory mediators, are able to inhibit the production of IL-12 by dendritic cells and modulate immunity against microorganisms.[59]

Eicosanoids are synthesized from a wide range of cells including leukocytes, vascular epithelial cells, and platelets. Although erythrocytes contain arachidonic acid (AA), they do not possess the oxidative enzymes required for AA metabolism.[60] Biosynthesis of eicosanoids depends on the availability of AA which is produced from membrane phospholipids by the action of phospholipase A_2. Arachidonic acid can be metabolized through three main pathways: the cyclooxygenase (COX) pathway producing the prostanoids; the lipoxygenase (LO) pathway producing the leukotrienes and lipoxins and the P-450 epoxygenase pathway producing hydroeicosatetraenoic acids (HETEs) and epoxides.[54] The pattern of eicosanoid production is determined in a stimulus and cell-specific fashion. All cell membranes of the eukaryote contain a rich source of phospholipids and AA, however some cells, the erythrocyte for example, lack COX and LO and therefore are incapable of production of most eicosanoids.

Transcellular biosynthesis is described as the cooperation of cells to produce eicosanoids: one cell type is activated to synthesize an intermediate compound, through a primary oxidative enzyme (COX or LO), that is transferred to a neighboring cell to carry out the final synthesis. The second cell does not need to be activated, but can convert the intermediate compound to the biologically active form by the presence of secondary enzymes such as prostaglandin isomerases, LTA_4 hydrolase or LTC_4 synthase. For example, LTA_4 produced by neutrophils can be converted into LTC_4 by vascular epithelium or into LTB_4 by erythrocytes. Also, PGI_2 is produced from platelet derived PGH_2 by vascular epithelium or lymphocytes.[54,60]

Multiple analytical techniques for quantitation of eicosanoids have been developed and include immunoassays such as enzyme-linked immunosorbent assay (ELISA) or radio-immunoassays (RIA), and gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS-MS).[61–63] Immunoassays provide excellent sensitivity but can only be used for the quantification of single analytes, and are susceptible to cross-reactions. These impairments are overcome by GC-MS or GC-MS-MS, but these methodologies are labor intense and time consuming due to the requirement of derivatization prior to analysis. LC-MS-MS based methods have become highly valuable in the measurement of various eicosanoids and other chemicals in biological samples because of their high sensitivity, high selectivity and simplicity of sample preparation.[64–67]

While there is little information in human literature and even less in veterinary literature regarding eicosanoids and stored blood products, some reports have documented that certain eicosanoids and other lipids accumulate with prolonged storage. In a previous study, our research group has demonstrated a significant increase in an array of eicosanoids during storage and transfusion of canine packed red blood cells.[68] Similarly, Jacobi *et al* demonstrated that prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) increased during storage, but stated these levels remained within physiologic limits and likely do not contribute to the risk of immunomodulated transfusion reactions.[69] Spinelli *et al*, demonstrated that PGE_2 and TXB_2 levels increased with storage, and that leukoreduction prior to storage significantly decreased TXB_2 accumulation and, to a lesser degree, PGE_2 as well.[70] Since leukocytes and platelets are

primarily responsible for the production of eicosanoids within stored blood products, it is reasonable to assume that leukoreduction may serve to decrease eicosanoid accumulation. Recently however, other human reports have linked accumulation of biologically active lipids, including arachidonic acid, in stored whole blood with transfusion-related acute lung injury (TRALI) even in units that have been leukoreduced.[15]

Transfusion-related acute lung injury has been linked to a number of mediators including biologically active lipids. Silliman *et al*, provide evidence that bioactive lipids that accumulate during storage may contribute to TRALI as a result of neutrophil priming. Lipids identified in that study included nonpolar lipids such as arachidonic acid, HETE, 12-HETE and 15-HETE. These lipids accumulate in red blood cell concentrates and therefore it is presumed they can be produced from peroxidation in addition to enzymatic conversion from arachidonic acid.[15] Lysophosphatidylcholine (lyso-PC) is another lipid derived from phospholipid membranes that can accumulate in storage and prime neutrophils. Both the non-polar lipids and lyso-PCs accumulate despite removing the platelet and leukocyte population and therefore the risk of TRALI is not completely diminished by the process of leukoreduction.[16,71] Although no veterinary studies have evaluated lipids and their role in transfusion reactions, it is reasonable to believe these lipids can accumulate in companion animal blood products as well. Further studies are needed to identify these lipids as well as to evaluate whether storage conditions can affect their concentrations.

Efforts to Reduce Storage Lesions

Storage lesions can be minimized by specific processing procedures either before storage or immediately prior to transfusion to the recipient. These procedures include leukoreduction prior to or after storage, post-storage cell washing, and rejuvenation prior to administration.[12] With leukoreduction prior to storage, the majority of leukocytes and platelets are extracted from the unit of blood via passage through a specialized filter, preventing these cells from influencing the environment within the unit and reducing the inflammatory response associated with blood transfusion in the recipient.[27] Pre-storage leukoreduction has been shown in veterinary medicine to effectively remove 99% of donor leukocytes.[72] Despite this, leukoreduction is not commonly performed in veterinary clinics. This is most likely due to decreased availability and a lack of knowledge regarding whether leukoreduction is beneficial in companion animals. In units of stored human packed red blood cells, the removal of leukocytes and platelets via specialized leukoreduction filters prior to storage results in a significant reduction in PS expression on erythrocytes.[40] In veterinary medicine, Herring *et al*, determined that PS expression on microparticles can be identified in canine whole blood, and that leukoreduction will attenuate the expression of PS on microparticles.[73] However, in that study, because microparticle PS expression was measured, it is unclear from which cell line PS originated. Currently, it is unknown how leukoreduction and storage will affect the expression of PS directly on the canine red blood cell. While leukoreduction removes the leukocyte and platelet population, it can still leave behind microparticles, free hemoglobin and inflammatory mediators. Additionally, levels of thromboxane B_2 can

increase during the leukoreduction process, which may be relevant when blood products are leukoreduced post-storage and immediately administered to the recipient.[74] This technique is no longer recommended in humans due to subsequent hypotension.[75] Alternatively, post-storage washing of RBCs prior to transfusion may help remove products left behind from leukoreduction. During the washing process, saline is added to erythrocyte units then the units are centrifuged and the supernatant is removed. Several human studies have proven the benefit of washing units prior to transfusion including a reduction in inflammatory markers.[76–80] Additional research is needed in veterinary medicine to assess the potential benefit of post-storage washing. Rejuvenation is another post-storage method developed to address storage lesions. During rejuvenation, a solution containing inosine, phosphate, and adenine is added to stored erythrocyte units prior to transfusion to restore RBC morphology, ATP, and 2,3 DPG concentrations. Rejuvenation appears to be beneficial in human research, however studies are lacking in companion animals.[81,82] Overall, strategies to minimize storage lesions have proven beneficial in human medicine. This may translate similarly to veterinary medicine however cost versus benefit must be considered in companion animals.

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CHAPTER III

Effect of leukoreduction and storage on erythrocyte phosphatidylserine expression and eicosanoid concentrations in units of canine packed red blood cells.

Introduction

Blood transfusions are commonly used for the treatment of anemia in critical veterinary patients. Storage of blood products, although necessary, creates an unnatural environment that can lead to accelerated product degradation.[1] In humans and dogs, a direct correlation has been established between blood product storage time and increased morbidity and mortality in transfused patients, suggesting that stored blood products can undergo significant changes that lead to an increased risk of complications in the recipient.[2-7] Numerous studies have investigated red blood cell storage lesions, and several mechanisms have been identified as contributors to the degradation of stored erythrocytes.[1,4,8]

In humans, an increase in phosphatidylserine (PS) expression on the red cell surface is one recognized storage lesion.[9-11] Phosphatidylserine is a negatively charged phospholipid, comprising a portion of the erythrocyte cell membrane. Normally, PS is confined to the inner leaflet of the erythrocyte cell membrane but, in aged or damaged erythrocytes, PS will translocate to the cell surface and serve as an indicator of erythrocyte quality. An increase in expression of erythrocyte PS will signal the need for cell removal via mononuclear phagocytic cells, potentially decreasing the life span of transfused erythrocytes. [9, 12-14]

Recently, it has been demonstrated that there is a significant accumulation of eicosanoids, such as prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), leukotriene B_4 (LTB_4), thromboxane B_2 (TXB_2), and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, a stable prostacyclin metabolite), in units of canine packed red blood cells (pRBCs) during storage and transfusion.[15] Eicosanoids are signaling molecules derived from arachidonic acid. These molecules are involved in a wide range of physiologic processes that include maintenance of vascular and bronchial tone, platelet aggregation, gastrointestinal motility, and renal blood flow. Additionally, eicosanoids can modulate the inflammatory response via the interaction with signaling molecules, cytokines and chemokines,[16] potentially contributing to the development of transfusion reactions. Although it is unknown how eicosanoids within blood products will affect the recipient during a transfusion, the infusion of prostacyclin in dogs has been shown to cause immediate and dramatic vasodilation.[17] Additionally, other eicosanoids within blood products, such as thromboxane, which causes platelet activation and vasoconstriction, could adversely affect the transfusion recipient.

Eicosanoids are synthesized from a wide range of cells, including leukocytes, vascular epithelial cells and platelets. Although erythrocytes contain arachidonic acid, they do not possess the oxidative enzymes involved in arachidonic acid metabolism and eicosanoid synthesis.[18] The leukocyte and platelet populations inside units of pRBCs are probably the major contributors to arachidonic acid metabolism.[15] In humans, leukocytes will lyse early in storage, releasing enzymes that can damage the erythrocyte, causing an

increase in PS expression, and contribute to the synthesis of proinflammatory molecules, including phospholipaseA_2, which can contribute to eicosanoid production.[4,19] With the use a leukoreduction filter prior to storage, the majority of leukocytes and platelets are extracted from the unit of blood, preventing these cells from influencing the environment inside the pRBC unit, and reducing the inflammatory response associated with transfusions.[20] In units of stored human pRBCs, the removal of leukocytes and platelets via leukoreduction prior to storage causes a significant reduction in erythrocyte PS expression.[10] Reduction in concentrations of leukocytes and platelets within the unit also removes the main source of eicosanoid synthesis.

Currently, it is unknown how leukoreduction and storage will affect erythrocyte PS expression and eicosanoid concentrations in units of canine pRBCs. The objective of this study was to determine the effects of leukoreduction on erythrocyte PS expression and eicosanoids that accumulate in units of canine pRBCs after storage and transfusion. Our hypothesis was that the use of a leukoreduction filter to eliminate the majority of leukocytes and platelets from pRBCs prior to storage would significantly decrease erythrocyte PS expression and eicosanoid concentrations.

Materials and Methods

Animals

Dogs were chosen from our research colony and included in the study if they were determined to have normal health status and they were not exposed to any medications or vaccines for at least 2 weeks prior to initiation of the study. Normal health status was established by detection of no abnormalities on physical examination, complete blood

count, serum chemistry, urinalysis, and heartworm and tick-borne disease testing. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee and was in compliance with the requirements of a facility accredited by the American Association for Accreditation of Laboratory Animal Care.

Blood Donation, Leukoreduction, and Sample Collection

The donors were randomly separated into one of two groups, a leukoreduction (LR) group and a non-leukoreduction (non-LR) group. Each dog underwent a standard blood donation. Briefly, the donors were positioned in either right or left lateral recumbency, and the fur overlying the jugular vein was clipped and the skin aseptically prepared. A 16-gauge needle was inserted into the jugular vein and approximately 450 milliliters of blood was collected aseptically, under negative pressure, into a standard triple blood banking bag[a] for the non-LR group and a quadruple blood banking bag[b], containing a leukoreduction filter, for the LR group. The units contained an anticoagulant citrate phosphate dextrose solution. There were no adverse events detected in the donor dogs during or after blood collection.

For the LR group, the unit was leukoreduced and platelet-depleted by passage of blood through a leukoreduction filter directly after collection. To assess the extent of leukoreduction, blood samples were collected before and after leukoreduction from the in-line tubing system for a total leukocyte and platelet count using an automated hematologic analyzer.[c] For both LR and non-LR groups, pRBCs were created by separating the red cells and plasma via centrifugation. To remove the plasma following centrifugation, external pressure was applied to the blood bag, and the plasma was passed

via a connecting tube into an attached empty bag for subsequent storage as fresh frozen plasma. While still containing plasma, the connecting tube between the two blood bags was sealed and removed. The plasma in the sealed tube was collected, snap frozen in liquid nitrogen, and stored at -80^{o}C. This plasma represented the initial sample (*"donation sample"*) for eicosanoid analysis. The remaining unit of pRBC was separated, to create two half units, by applying external pressure to the unit and allowing erythrocytes to pass, via a connecting tube, into an attached empty bag. While still containing red blood cells, the connecting tube between the two blood bags was sealed and used as the initial sample (*"donation sample"*) for PS expression. Each half unit of pRBCs was stored vertically at 4^{o}C in a dedicated refrigerator for 10 or 21 days. The donors underwent at least a 28-day recovery period after blood collection, switched groups and the process was repeated.

On Day 10 of storage, one half unit from each dog (4 LR and 4 non-LR) was removed and samples were collected for analysis. When removed from refrigeration, each half unit was infused with 50 mL of 0.9% saline, mixed gently, and 1 mL of reconstituted packed red blood cells was removed from the unit for measure of PS expression. An additional 20 mL of reconstituted packed red blood cells were collected, centrifuged, and the supernatant was removed for eicosanoid analysis. This sample was snap frozen in liquid nitrogen and stored at -80^{o}C until analysis. The collected erythrocytes and supernatant represented the "*Day 10 pre-transfusion*" samples. To mimic transfusion conditions, the remainder of each half unit was left at room temperature for 5 hours. At the completion of this time period, using the same procedures

as mentioned above, a 1 mL sample was collected and processed for the measurement of erythrocyte PS expression, and a 20 mL sample was collected for eicosanoid analysis. The collected erythrocytes and supernatant represented the "*Day 10 post-transfusion*" samples. On Day 21 of storage, the 8 half units of pRBCs that had remained in refrigeration were removed and processed similar to the Day 10 samples. The erythrocytes and supernatant collected on Day 21 of storage represented the "*Day 21 pre-transfusion*" and "*Day 21 post-transfusion"* samples.

Phosphatidylserine Analysis

A flow cytometric assay was used to quantitate the expression of PS on canine erythrocytes. Labeling of cells was performed based on a previously described protocol.[21] Personnel performing flow cytometry were blinded to knowledge of whether samples were LR or non-LR. For red blood cell preparation, 50 µL of concentrated erythrocytes were washed twice with FACS-PBS, containing 0.2% bovine serum albumin[d], and 10 µL of the washed red blood cells were resuspended in 90 µL of FACS-PBS. For flow cytometric analysis, 16 µL of washed RBCs were incubated with annexin-V-FITC[e] and annexin binding buffer for 15 minutes in the dark at room temperature. After incubation, 400 µL of annexin binding buffer was added, gently mixed, and analyzed within one hour. Flow cytometric analysis was performed with a flow cytometer[f] and computer software.[g] Red blood cell populations were displayed on log forward-scatter versus log side-angle light scatter plots. Gates were adjusted to baseline erythrocyte populations, and 5,000 gated events were recorded for each labeling. For quality control, both positive and negative controls were included. Expression was

quantified by the intensity of annexin-V-FITC fluorescence and expressed as median fluorescence intensity (MFI).

Eicosanoid Analysis

Using a previously established technique,[22] the concentrations of arachidonic acid (AA), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), $PGF_{2\alpha}$, TXB_2 (a stable metabolite of thromboxane A_2), 6-keto-$PGF_{1\alpha}$ (a stable, hydrolyzed product of prostacyclin (PGI_2)) and LTB_4 were analyzed by liquid chromatography-mass spectrometry. Personnel performing spectrometry were blinded to knowledge of whether samples were LR or non-LR. The thawed plasma/saline supernatant, which contained deuterated internal standards (d_4-8-iso $PGF_{2\alpha}$, d_4-LTB_4, and d_8-arachidonic acid), were extracted by using C18 SepPak columns.[h] After drying, 10 µl of the resolubilized lipids was injected onto an Acquity UPLC BEH C18 column (1.7 µm, 100 ×2.1mm I.D.).[i] The analytes were eluted from the analytical column with a gradient program and directed into a mass spectrometer.[j] The concentrations were determined by measuring the area under the chromatographic peak and comparing this value to the area under the chromatographic peak for the internal standard. A computer software program[k] was employed for data acquisition and processing. The eicosanoid concentrations were normalized to the volume of plasma used for analysis and expressed as pmol/ml plasma. The estimated limits of detection with this protocol are between 0.1-10 nM on-column.

Statistical Analysis

Sample size calculation was performed based on previously published data.[15] The assumptions used in the calculations were an alpha of 0.05 and a power of 0.95. An

estimated sample size of 8 dogs would detect if the concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2 following leukoreduction would be similar to the initial donation sample. A linear mixed model was fit with PROC MIXED in a statistical software program[l] for the each outcome. Run, sequence, filter, sample, and the filter and sample interaction were included as fixed effects with a Kenward-Rogers degrees of freedom method specified. Dog identity was included as random effect with a variance component covariance structure specified. Repeated measures of dog identity within run for the different samples were specified in a repeated statement with a spatial power law covariance structure. The interaction term was dropped from the model if it was not significant. If the interaction term was significant, differences in least squares means between each of the concentrations of one variable were calculated for each concentration of the other variable in the interaction using an LSMESTIMATE statement. The simulate adjustment for multiple comparisons was used for significant effects. The distribution of the conditional residuals was evaluated for each outcome to ensure the assumptions of the statistical model had been met. An alpha level of 0.05 was used to determine statistical significance for all methods.

Results

Animals

Eight healthy adult research Walker Hound dogs, five males and three females, were used in this study. The mean age of the dogs was 1.5 years (range, 1.5 to 6.5 years), and their mean body weight was 27.4 kg (range, 20.5 to 30.5 kg).

Leukoreduction

The leukocyte and platelet counts prior to and after leukoreduction are represented in Figure 1. Leukoreduction was effective at removing all leukocytes, however, although leukoreduction was effective at removing 98.3% of the platelets in 7 of the 8 units, but there was only a partial reduction in platelet count (59.7%) in one unit.

Phosphatidylserine Expression

The MFI of erythrocyte PS expression is summarized in Table 1. Compared to the respective donation sample, there was no significant changes in the MFI at any time point for the non-LR units and the LR units. Additionally, when comparing the MFI between the non-LR and LR units, there were no significant differences at any time points.

Eicosanoid Concentration

The eicosanoid concentrations for the non-LR and LR units at all time points are represented in Table 2. There was no difference in AA, PGE_2, PGD_2, and LTB_4 concentrations at any time point, nor was there a difference in the concentrations between non-LR and LR units.

At the time of donation, the $PGF_{2\alpha}$ concentrations in LR units were significantly ($p<0.0001$) greater than non-LR units. $PGF_{2\alpha}$ concentrations in LR units then dropped rapidly with storage, such that there was no difference in $PGF_{2\alpha}$ concentration between the LR and non-LR units for any of the remaining time points. For the LR-units, the $PGF_{2\alpha}$ concentration in all of the subsequent samples, pre and post transfusion, were significantly ($p<0.01$) less than the initial donation sample. There was no significant

difference in $PGF_{2\alpha}$ concentration among the pre and post transfusion samples collected from the LR units on Days 10 and 21. There was no difference in $PGF_{2\alpha}$ concentration among the samples collected from the non-LR units.

At the time of donation, the TXB_2 concentrations in LR units were significantly ($p<0.0001$) greater than non-LR units. TXB_2 concentrations in LR units then dropped rapidly with storage, such that there was no difference in TXB_2 concentration between the LR and non-LR units for any of the remaining time points. The TXB_2 concentration in all LR samples, except for Day 10 post-transfusion, was significantly ($p<0.05$) decreased compared to the donation sample. There was no significant difference in TXB_2 concentration among the pre and post transfusion samples collected from the LR units on Days 10 and 21. There was no difference in TXB_2 concentration among the samples collected from the non-LR units.

When compared to the initial donation sample, there was a significant ($p<0.0001$) increase in 6-keto-$PGF_{1\alpha}$ concentrations irrespective of unit type for both LR and non-LR units on both Days 10 and 21 for both the pre and post transfusion samples. The day 10 post transfusion sample concentrations were significantly greater ($p=0.0029$) than the day 21 pre transfusion samples, irrespective of unit type. The 6-keto-$PGF_{1\alpha}$ concentrations for the LR units were significantly increased ($p=0.0163$) compared to the non-LR unit concentrations, irrespective of sample time.

Discussion

Transfusion with stored blood products has been associated with an increase in morbidity and mortality in transfused patients, compared to transfusion with fresh

products.[2-8] In human medicine, increased PS expression on the red blood cell surface and the accumulation of eicosanoids inside the stored unit are proposed mechanisms that contribute to the degradation of stored erythrocytes and transfusion reactions.[9-11, 23] With the use a leukoreduction filter prior to storage, removing the majority of the leukocytes and platelets from the unit, the environment inside the unit is thought to be less conducive for the formation of storage lesions, which is proposed to reduce the inflammatory response and reactions associated with transfusions.[20] The results of our study demonstrate that, in units of canine pRBCs, leukoreduction and storage have minimal impact of erythrocyte PS expression, but alter concentrations of some eicosanoids.

Based on our study, erythrocyte PS expression was not altered by leukoreduction, length of storage, or simulated transfusion. Similar findings have been reported in human studies, in which only a small percentage of PS-positive erythrocytes (3.5 to 4.5%) were detected after prolonged storage times of up to 7 weeks using similar techniques.[11,24-26] One possible explanation for why an increase in RBC PS expression was not detected is that the PS positive portions of the erythrocyte membrane were released from the cell and became microparticles in the unit. In stored units of human pRBCs, erythrocytes can undergo vesiculation to return to a PS-negative state by shedding PS-containing vesicles.[9,27] It is unknown when stored erythrocytes maximally express PS, but PS expression probably occurs continuously, followed by vesicle formation. In a previous study evaluating units of canine RBC concentrates, PS expression on the surface of microparticles increased during storage.[28] Therefore, if the PS-positive microparticles in units of canine pRBCs were released from erythrocytes, then the PS expression on

individual red blood cells would have minimal change during leukoreduction, storage and transfusion.

In humans, Burger et al demonstrated an increased erythrocyte PS expression during storage of blood products, but this expression was transient and associated with increasing amounts of PS-positive vesicle formation. Additionally, the LR units were stored for 42 days, twice as long as our study, and the PS expression only changed after an overnight incubation at 37°C, to mimic the environment of red blood cells following a transfusion.[9] In our study, the units were subjected to 5 hours at room temperature (approximately 21°C) to simulate the temperature change during a transfusion before the erythrocytes enter the recipient, but we did not evaluate PS expression on erythrocytes at a temperature that would mimic the body temperature of the recipient. It is possible that with a longer storage time and greater erythrocyte incubation temperature, to mimic the environment of transfused erythrocyte, we could have detected a change in PS expression on RBCs. Although PS translocation is a well-described storage lesion in humans,[9-11] due to unpredictable vesiculation and the difficulty in determining microparticle origin, using PS expression as a marker for erythrocyte quality in units of canine pRBCs can be unpredictable and difficult to interpret.

In a previous study performed in dogs, storage of units of pRBCs caused a decrease in arachidonic acid concentration, followed by a progressive increase in the concentration of eicosanoids inside the units.[15] One possible explanation for these changes in eicosanoids during storage was a decline of cell health, particularly in leukocytes and platelets, leading to a decrease in cell viability and an increase in

enzymatic conversion of arachidonic acid to the various eicosanoids. In humans, the pre-storage removal of leukocytes and platelets via leukoreduction decreases the concentrations of PGE_2 and TXB_2 in stored units of pRBCs.[29] In contrast, in our study, the removal of leukocytes and platelets prior to storage did not significantly decrease eicosanoid concentrations.. At most sample time points in our study, eicosanoid concentrations were similar between LR and non-LR units, suggesting that leukoreduction may have minimal impact on the accumulation of eicosanoids in units of canine pRBCs.

Compared to non-leukoreduced units, the passing of blood through the leukoreduction filter prior to storage created an immediate and marked increase in TXB_2 and $PGF_{2\alpha}$ concentrations. The increase in TXB_2 concentration is particularly concerning because, as a potent platelet activator and vasoconstrictor, an increase in this eicosanoid could have a significant impact on the hemodynamic stability of transfusion recipients. This concern, however, may only be applicable to blood products that are leukoreduced and immediately administered to the recipient because, during storage, TXB_2 concentrations in LR units decreased to concentrations similar to non-LR units. It is unknown for how long after leukoreduction TXB_2 concentrations remain elevated but, after 10 days of storage, the concentrations are similar to non-LR units.

A possible explanation for the increase in TXB_2 concentration immediately following leukoreduction is that platelets are activated as they adhere to the fibers within the filter. In human blood products, based on platelet shape change and pseudopod formation, platelets appear to become activated as they pass through leukoreduction

filters.[30] Once activated, platelets will increase expression of integrins needed for aggregation, change shape to cover a greater surface area, empty the contents of granules, and release arachidonic acid to be converted to thromboxane A_2.[31] After synthesis, thromboxane A_2 exhibits a very short half-life and is non-enzymatically decomposed to the stable metabolite TXB_2.[32] While platelets are the primary source of thromboxane A_2, several other cells are capable of synthesizing this eicosanoid, including leukocytes and endothelial cells.[32,33] Even though the cyclooxygenase (COX)-2 enzyme has been identified in canine platelets,[34] COX-1 is the primary isoform in platelets, and probably responsible for the increased synthesis of TXB_2 in units of pRBCs. It is unknown how elevated concentrations of TXB_2 affect the patient, but the increased TXB_2 associated with leukoreduction and immediate transfusion may have the potential to adversely impact the recipient.

In addition to TXB_2, $PGF_{2\alpha}$ also significantly increased immediately after leukoreduction. Prostaglandin $F_{2\alpha}$, is synthesized via three pathways from PGE_2, PGD_2, or PGH_2 by PGE 9-ketoreductase, PGD 11-ketoreductase, or PGH 9-,11-endoperoxide reductase, respectively.[35] Collectively referred to as prostaglandin F synthases (PGFS), these enzymes are expressed in numerous organs including the liver, lung, brain, kidneys and uterus, but PGFS mRNA has also been identified in peripheral blood lymphocytes.[36] The mechanism of increased $PGF_{2\alpha}$ after leukoreduction in this study is unclear, but it is possible that PGFS were released from lymphocytes activated during the filtration process. Some studies have described leukocyte activation with the use of leukocyte depletion filters,[37-39] however, these findings were not consistently replicated in other

leukoreduction studies.[40-43] Furthermore, leukocyte rupture is possible with prolonged filtration, resulting in free enzymes and other cellular components.[44] Similar to TXB_2, the increase in $PGF_{2\alpha}$ concentration following leukoreduction is particularly concerning because $PGF_{2\alpha}$ is associated with vasoconstriction and bronchoconstriction, which could impact the hemodynamic stability of transfusion recipients.[16]

The decrease in TXB_2 and $PGF_{2\alpha}$ concentrations during storage could be associated with the short half-life of these molecules. Prostanoid activity is usually short lived, and catabolism of these molecules occurs rapidly and primarily through the pulmonary circulation. Prostanoid catabolism involves several enzymes, particularly 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which results in oxidation of the 15-OH group to the corresponding ketone.[27,45,46] In addition to being found in high concentrations in the lungs, 15-PGDH is located throughout the body, including a high expression in leukocytes.[47] Despite removal of the leukocyte population, the release of 15-PGDH from activated leukocytes at the time of leukoreduction may have contributed to TXB_2 and $PGF_{2\alpha}$ catabolism.

Compared to our previous study,[15] despite some similar trends, overall the eicosanoid concentrations in this current study were significantly lower. One potential explanations for these differences include quantity of cells within the unit. Unlike the previous study which used greyhounds as donor dogs, our study used hound dogs which, compared to greyhounds, have a lower percentage of circulating erythrocytes. The average hematocrit from the dogs in the current study was 49.5%, while the average PCV

in our previous study was 68%. The lower percentage of cells inside the units could have contributed to this decrease in eicosanoid synthesis.

Biosynthesis of eicosanoids depends on the availability of arachidonic acid, which is derived from membrane phospholipids by several enzymes, including phospholipase A_2. Once released, arachidonic acid can be metabolized by three main pathways: the COX pathway that produces prostanoids, the lipoxygenase (LOX) pathway that produces leukotrienes and lipoxins, and the P-450 epoxygenase pathway that produces epoxides.[16] The erythrocyte membrane is a rich source of arachidonic acid, but lacks both the COX and LOX enzymes, and therefore cannot produce the majority of eicosanoids.[18] However, the arachidonic acid-rich erythrocytes can contribute to eicosanoid synthesis via transcellular biosynthesis, the cooperation among different cell types to produce eicosanoids. During transcellular biosynthesis, one cell type can synthesize an intermediate compound, through a primary oxidative enzyme, which is transferred to a neighboring cell to complete the final synthesis.

In our study, the only eicosanoid that increased in concentration during storage or simulated transfusion was 6-keto-$PGF_{1\alpha}$. This was similar to a previous study that identified an increase in 6-keto-$PGF_{1\alpha}$ concentration in units of canine pRBCs during storage and transfusion.[15] This prostaglandin is produced via non-enzymatic conversion from PGI_2, which is derived by the vascular epithelium or lymphocytes.[16] Interestingly, after the removal of the leukocytes and platelet via leukoreduction, the predominate cell type expected to be remaining in the unit is the erythrocyte, which is not capable of synthesizing prostanoids. Therefore, we presume that, despite leukoreduction,

components within units of pRBCs contributed to increased concentrations of 6-keto-$PGF_{1\alpha}$ via a process similar to transcellular biosynthesis. Specifically, arachidonic acid could be derived from the rich phospholipid bilayer of erythrocytes, and converted to prostacyclin via the COX-2 enzymes found in other cellular components within units of pRBCs.

While it is unknown how the accumulation of eicosanoids in units of pRBCs will affect physiologic responses within the transfusion recipient, considering the functions of these molecules, the administration of blood products with elevated concentrations of eicosanoids could have detrimental effects. For example, thromboxane promotes platelet aggregation, vasoconstriction and bronchoconstriction, all of which may adversely affect hypercoagulable and hypertensive patients.[16] Thromboxane can also enhance the production of IL-8, a pro-inflammatory cytokine and potent neutrophil chemoattractant[48,49] and, therefore, may promote transfusion-associated inflammatory responses. Additionally, transfusing blood products with high concentrations of prostacyclin, which inhibits platelet aggregation and promotes vasodilation,[16] may further exacerbate complications associated with systemic hypotension in critical patients. Although our study did not detect significant accumulation of eicosanoids, further studies under a range of different storage conditions are needed to determine if eicosanoids can accumulate in storage to a concentration significant enough to cause adverse effects in the recipient.

To determine the effectiveness of leukoreduction, leukocyte and platelet counts were performed immediately before and after leukoreduction. The filter was effective at

removing all leukocytes, but the filters were less effective at removing platelets prior to storage. Seven units had a post-filter platelet count of less than 20 K/µL, but one unit had 137 K/µL platelets post filtration, a 60% reduction in platelet count compared to the pre-filtration sample. This elevated platelet count in the LR units could have altered the results of our study. If the eicosanoid and phosphatidylserine results from this unit were removed from our analyses, however, there was no major change in overall results, and the results from this unit were therefore included in our final analyses.

There were several limitations with this study. One limitation was not using a transfusion administration set during the simulated transfusion. With the use of a transfusion administration set and the passing of blood through an additional in-line administration filter, additional changes could have developed in either eicosanoid concentrations or PS expression. Another limitation of this study was the storage length of 21 days. Compared to other studies investigating storage lesions, a 21-day storage period may have been too short of time to detect a change in eicosanoid concentration or PS expression, and it is possible that a longer storage duration would be associated with greater changes. Additionally, our study evaluated the prostanoids derived from the COX pathway and LTB4 produced by the LOX pathway, but the epoxides synthesized by the P-450 pathway were not measured. Even though leukoreduction did not significant reduce the eicosanoids measured in this study, it is possible that the removal of leukocytes and platelets prior to storage could have reduced vasoactive epoxides. Finally, despite a sample size calculation that suggested that 8 dogs would provide ample

power for this study, including more dogs in this study may have provided additional results.

Our study demonstrated that PS expression on the surface of erythrocytes was not affected by leukoreduction or storage duration. Additionally, the passage of blood through a leukoreduction filter causes an immediate and dramatic increase in TXB_2 and $PGF_{2\alpha}$ concentrations, but these concentrations then decrease during subsequent storage. Despite leukoreduction, the concentration of 6-keto-$PGF_{1\alpha}$ continued to increase during storage and simulated transfusion. Overall, when compared to non-LR units, the addition of a leukoreduction step prior to storage had a minimal impact on the accumulation of eicosanoids in canine units of pRBCs. While leukoreduction may be beneficial in other aspects of transfusion medicine, based on the results in this study, using leukoreduction to decrease PS expression and eicosanoid concentrations does not appear to be effective.

Footnotes

a. Teruflex Optisol Triple Collection Blood Bag, Terumo Corporation, Tokyo, Japan

b. Imuflex-WB-RP Blood Bag with integral whole blood leukocyte reduction filter, Terumo Corporation, Tokyo, Japan

c. Abbott Cell-Dyn® 3700, Abbott Laboratories, Abbott Park, IL

d. Bovine Serum Albumin, Sigma-Aldrich, St. Louis, MO

e. FITC Annexin V/Dead Cell Apoptosis Kit, Life Technologies, Grand Island, NY

f. FACSCalibur, BD Biosciences, San Jose, CA

g. CellQuest Pro software, BD Biosciences, San Jose, CA

h. HyperSep Retain PEP 60 mg, 1mL, Thermo Fisher Scientific, Waltham, MA

i. Acquity UPLC BEH C18 column, Waters Corporation, Milford, MA

j. TSQ Quantum Access Max, Thermo Fisher Scientific Inc, San Jose, CA

k. Xcaliber software, ThermoFisher Scientific Inc, San Jose, CA

l. SAS for Windows version 9.4, SAS Institute, Inc., Cary, NC

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Figure 3.1 Leukocyte (A) and platelet (B) counts prior to and after leukoreduction. LR, Leukoreduced

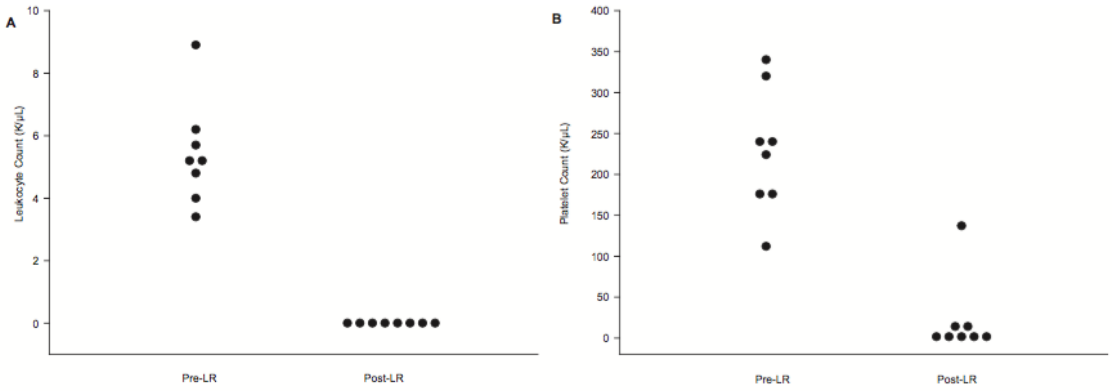

Table 3.1 Based on flow cytometric analysis, the MFI (mean ± standard deviation) of canine erythrocyte PS expression, with and without leukoreduction, at collection (donation) and after various lengths of storage (pre-transfusion) and after a simulated transfusion (post-transfusion).

Sample	**Non-LR Units of pRBCs**	**LR Units of pRBCs**
Day 0 - Donation	7.74±1.04	7.57±0.83
Day 10 – Pre-transfusion	7.28±1.10	7.21±0.68
Day 10 – Post transfusion	7.13±0.93	6.99±1.25
Day 21 – Pre transfusion	7.88±0.93	7.85±0.98
Day 21 – Post transfusion	7.42±1.59	7.77±1.28

MFI, median fluorescence intensity; PS, phosphatidylserine; LR, leukoreduced

Table 3.2 The eicosanoid concentration (nM) (mean ± standard deviation) in units of canine pRBCs, with and without leukoreduction, at collection (donation) and after various lengths of storage (pre-transfusion) and after a simulated transfusion (post-transfusion).

nM, nanomolar; pRBCs, packed red blood cells; Non-LR, non-leukoreduced; LR, leukoreduced; AA, arachidonic acid; PGE_2 prostaglandin E_2; PGD_2, prostaglandin D_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TXB_2 thromboxane B_2; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; LTB_4, leukotriene B_4.
Results labeled "*" illustrate significant ($p \leq 0.05$) differences from the respective donation sample.
Results labeled "†" illustrate significant ($p \leq 0.05$) differences between Non-LR and LR samples at the same time point.

Eicosanoid	Day 0 - Donation		Day 10 - Pre transfusion		Day 10 – Post transfusion		Day 21 - Pre transfusion		Day 21 - Post transfusion	
	Non-LR	LR	Non-LR	LR	Non-LR	LR	Non-LR	LR	Non-LR	LR
AA	735.9±258.2	586.5±277.2	398.3±162.8	783.7±427	413.7±282.4	715.6±290	431.9±246	544±357.7	578.9±493.2	514.5±285.7
PGE_2	0.6±1.3	1.0±0.7	0.8±1.2	0.2±0.1	1.0±2.4	0.3±0.2	0.6±0.6	0.1±0.1	0.4±0.5	0.3±0.4
PGD_2	0.5±1	0.3±0.2	0.7±1	0.1±0.1	0.9±2	0.1±0.1	0.4±0.5	0.1±0.1	0.4±0.5	0.2±0.3
$PGF_{2\alpha}$	0.2±0.3†	1.8±0.9†	0.4±0.3	0.7±0.3*	0.7±0.9	0.9±0.4*	0.1±0.1	0.4±0.1*	0.2±0.2	0.4±0.3*
TXB_2	7.8±11.3†	123.1±44.6†	34±45.4	47.3±19.4*	60±127.8	64.4±22.4	12.1±16	21.5±9.6*	10.3±12.1	26.2±11.2*
6-keto-$PGF_{1\alpha}$	0.7±0.4	1.2±0.5	5.9±2.2*	6.2±2.4*	5.7±2.4*	8.4±1.3*	4.8±1.1*	5.3±1.1*	5.5±1.5*	6.4±1.5*
LTB_4	9.3±8.7	10±2.5	23.7±28.9	15.9±5.2	19.7±29.4	16±4.9	18.7±13.7	14.2±5.5	17.3±10.9	17.6±9.4

CHAPTER IV

CONCLUSION

In this study, units of packed red blood cells were evaluated for changes in both phosphatidylserine expression and eicosanoid concentrations in response to leukoreduction and various storage times. The results of our study demonstrate that, in units of canine pRBCs, leukoreduction and storage have minimal impact on erythrocyte PS expression, but alter concentrations of some eicosanoids. Data obtained from this study have resulted in new ideas regarding future directions for research surrounding storage lesions.

Significant changes in PS expression on erythrocytes were not noted in this study. As previously mentioned, similar findings have been reported in human studies, in which only a small percentage of PS-positive erythrocytes (3.5 to 4.5%) were detected after prolonged storage times of up to 7 weeks using similar techniques.[1-4] One possible explanation for why an increase in RBC PS expression was not detected is that the PS positive portions of the erythrocyte membrane were released from the cell, becoming PS expressing microparticles in the unit. In stored units of human pRBCs, erythrocytes can undergo vesiculation to return to a PS-negative state by shedding PS-containing vesicles.[5,6] It is unknown when stored canine erythrocytes maximally express PS, but PS expression may occur continuously, followed by vesicle formation. In a previous study evaluating units of canine RBC concentrates, PS expression on the surface of

microparticles increased during storage.[7] Therefore, if the PS-positive microparticles in units of canine pRBCs were released from erythrocytes, then the PS expression on individual red blood cells would have minimal change during leukoreduction, storage and transfusion. Future studies investigating both erythrocyte and microparticle PS expression concurrently in units of canine packed red blood cells would be helpful in clarifying this process.

In humans, Burger *et al* demonstrated an increased erythrocyte PS expression during storage of blood products, but this expression was transient and associated with increasing amounts of PS-positive vesicle formation. Additionally, the leukoreduced units were stored for 42 days, twice as long as our study, and the PS expression only changed after an overnight incubation at 37°C, to mimic the environment of red blood cells following a transfusion.[5] In our study, the units were subjected to 5 hours at room temperature (approximately 21°C) to simulate the temperature change during a transfusion before the erythrocytes enter the recipient, but we did not evaluate PS expression on erythrocytes at a temperature that would mimic the body temperature of the recipient. Future studies that incorporate a longer storage time and greater erythrocyte incubation temperature, to mimic the environment of transfused erythrocyte, are needed to potentially detect a change in PS expression on RBCs. Although PS translocation is a well-described storage lesion in humans,[1,5,8] due to unpredictable vesiculation and the difficulty in determining microparticle origin, using PS expression as a marker for erythrocyte quality in units of canine pRBCs can be unpredictable and difficult to interpret.

In humans, the pre-storage removal of leukocytes and platelets via leukoreduction decreases the concentrations of PGE_2 and TXB_2 in stored units of pRBCs.[9] In contrast, in our study, the removal of leukocytes and platelets prior to storage did not significantly decrease eicosanoid concentrations. Alternatively, we observed a marked increase, specifically in TXB_2, immediately post-leukoreduction, which raises concern regarding hemodynamic effects in the recipient. Furthermore, storage resulted in an increase in 6-keto-PGF1α, a stable prostacyclin metabolite. Transfusing blood products with high concentrations of prostacyclin, which inhibits platelet aggregation and promotes vasodilation, may further exacerbate complications associated with systemic hypotension in critical patients. However, it is unknown if the metabolites of thromboxane and prostacyclin are representative of the biologically active compound. Futhermore, it is unknown if these metabolites would have an effect on the recipient if transfused in high concentrations. Further studies under a range of different storage conditions are needed to determine if eicosanoids can accumulate in storage to a concentration significant enough to cause adverse effects in the recipient.

The exploration of the clinical significance of storage lesions in veterinary medicine is quite limited. Large observational studies of human patients, especially those with trauma, after cardiac surgery, or in a pediatric intensive care unit, found associations between transfusions of older PRBCs and increased rates of sepsis, multiple organ dysfunction syndrome, mortality or some combination of these.[10-12] Such studies raise concerns regarding whether older pRBC units should be used in critically ill patient populations. In a retrospective study summarizing data from 3,095 dogs on the

association of pRBC storage duration on morbidity and mortality, the age of transfused pRBCs did not contribute to mortality in the general canine population, although there may be associations with multiple organ dysfunction and coagulopathies.[13] Additionally, administration of older stored autologous RBCs has been shown to induce an inflammatory response in healthy dogs.[14]

In this study, we identified changes in canine pRBC units in response to leukoreduction and storage, however, we do not know if these changes would significantly affect patients to which these units were transfused. The next step in our research should involve prospective investigation of the recipient to which stored leukoreduced and non-leukoreduced allogenic units transfused. Subsequent monitoring of the recipient along with analysis of blood samples for various procoagulant and inflammatory markers would provide very useful information for veterinary transfusion medicine.

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