The Effects of Haemorrhagic Shock and Leukoreduction on Cytokine Accumulation in Canine Packed Red Blood Cells

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This thesis is presented for the degree of Research Masters with Training (RMT) of Murdoch University 2016
Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Chapters three and four are for publication in scientific journals. I am the primary author of these manuscripts, although they were written under the guidance of my principle supervisor and co-authors. The study design and experimental research were primarily undertaken by myself with assistance from my principle supervisor and other co-authors.

Chapter three has been published in the American Journal of Veterinary Research, and copyright for publication in this thesis has been sought. Chapter four has been accepted for publication in the Australian Veterinary Journal, and this chapter is consistent with the most recent copy submitted at the time of thesis submission. As a result of this, abbreviations may differ to the remaining thesis, and spelling in the third chapter is in American English.

Ethical approval for the experimental research in chapters three and four of this thesis has been granted by Murdoch University Animal Ethics Committee with approval number R2564/13.

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Abstract

Blood transfusions can be life-saving, however, adverse effects occur. Some adverse effects are attributed to cytokine accumulation in blood products during storage. Interleukin-8 (IL-8), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α), are cytokines that have been shown to accumulate during storage in human packed red blood cells (PRBC). Leukoreduction removes leukocytes from blood products and thus prevents cytokine accumulation. A recent study in dogs showed that IL-8 accumulated in canine PRBC collected from non-anaesthetised dogs, and leukoreduction prevented this accumulation. At our institution, blood is collected from anaesthetised terminal donors in haemorrhagic shock. We were thus interested to investigate the effect of this method of blood collection on cytokine accumulation in stored canine PRBCs. Acute haemorrhage in rats, pigs and people has been shown to increase inflammatory cytokines. It is unknown if similar increases occur in dogs with haemorrhagic shock, or if these cytokines are increased in blood collected from terminal donors. Therefore, we collected three 450 mL units of blood from anaesthetised dogs prior to euthanasia. We studied the first units (U1) and third units (U3) collected. Half of each unit was leukoreduced (LR) to produce one LR, and one non-leukoreduced (NLR) unit. Samples were collected from these units during storage on days 0, 10, 20, 30, and 37. Concentrations of IL-8, and TNF-α were assessed by the authors using our in-house multiplexed genomic and proteomic biomarker analyser. Concentrations of IL-1β were assessed by the authors using an in-house ELISA. In U1, collected prior to the onset of haemorrhagic shock, and in U3 collected after the onset of haemorrhagic shock, the concentration of IL-8 in NLR units was significantly greater than in LR units and increased during storage. Interleukin-1β did not significantly increase over time, and TNF-α was undetectable. Analysis of U3 compared with U1 did not show a significant difference in IL-8 concentration. Leukoreduction was successful at preventing accumulation of IL-8 in all units. Our research showed that IL-8 accumulates in canine PRBC during storage and
leukoreduction prevents this accumulation. Units collected from terminal donors in shock did not contain significantly more IL-8, and therefore may not lead to increased adverse reactions when administered to patients.
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I would also like to thank Dorian Lara for his assistance with blood collection and processing, and to Professor Peter Irwin, and Associate Professor David Miller for the use of their laboratory space and equipment.
1. Chapter 1: Introduction, Objectives and Hypotheses

1.1. Introduction

The aims of this project were to evaluate the concentration of cytokines in canine packed red blood cells (PRBC) during storage, to determine if cytokine concentrations are affected by the development of acute haemorrhagic shock in terminal canine blood donors, and to demonstrate the effect of leukoreduction on cytokine concentration in canine PRBC. The first study measured interleukin-8 (IL-8), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) during storage in non-leukoreduced (NLR) and leukoreduced (LR) canine PRBC, collected under anaesthesia prior to the onset of haemorrhagic shock. The second study measured the same cytokines during storage in NLR and LR canine PRBC, collected from terminal blood donors after the onset of haemorrhagic shock. Both studies evaluated the concentration of cytokines in stored canine blood products that had been leukoreduced prior to storage.

The second chapter of this thesis outlines the currently available literature, and summarises the pathophysiology of anaemia and associated transfusion, principles of blood collection and storage, pre-transfusion testing and administration, the changes that occur within blood products during storage, cytokines associated with transfusion, the adverse effects associated with the administration of cytokines, and lastly the effects of leukoreduction. Chapters three and four of this thesis are manuscripts that have been accepted for publication in scientific journals. I am the primary author of these manuscripts, although they were written under the guidance of my principle supervisor and co-authors. The study design and experimental research were primarily undertaken by me with assistance from my principle supervisor and other co-authors (see appendix ‘A’).
Chapter three has been published in the American Journal of Veterinary Research, and copyright for publication in this thesis has been sought. Chapter four has been accepted for publication in the Australian Veterinary Journal, and this chapter is consistent with the most recent copy submitted at the time of thesis submission. As a result of this, abbreviations may differ to the remaining thesis, and spelling in the third chapter is in American English.

1.2. Objectives

- To measure IL-8, IL-1β, and TNF-α at select time points during storage in canine PRBC collected from anaesthetised euvalaemic greyhounds (Chapter 3).
- To measure IL-8, IL-1β, and TNF-α during storage in canine PRBC collected from anaesthetised greyhounds in haemorrhagic shock, and compare these to cytokine concentrations in stored PRBCs from anaesthetized euvalaemic greyhounds (Chapter 4).
- To measure cytokines IL-8, IL-1β, and TNF-α during storage in canine PRBC leukoreduced prior to storage (Chapter 3 & 4).

1.3. Hypotheses

We hypothesised that IL-8, IL-1β, and TNF-α would increase throughout storage in canine PRBC collected from anaesthetised euvalaemic dogs. We further hypothesised that PRBC collected from terminal donors in haemorrhagic shock would have higher concentrations of IL-8, IL-1β, and TNF-α, compared with units from euvalaemic dogs. Lastly, we hypothesised that leukoreduction would prevent accumulation of IL-8, IL-1β, and TNF-α in all units of canine PRBC throughout storage.
Chapter 2: Literature Review

2.1. Red blood cell physiology, anaemia, and transfusion

The main function of red blood cells (RBCs) is to deliver oxygen to tissues. Anaemia results in a reduction of red blood cell (RBC) mass and therefore arterial oxygen content \((\text{CaO}_2)\). Severe anaemia can decrease tissue oxygen delivery \((\text{DO}_2)\) to a level that causes tissue hypoxia. Transfusion of RBCs improves \(\text{CaO}_2\), and therefore \(\text{DO}_2\), but is not without risk of adverse effects.

2.1.1 The role of the red blood cell in oxygen delivery

The primary role of RBCs is to carry oxygen from the pulmonary capillaries to the tissues and carbon dioxide from the tissues to the pulmonary capillaries. More than 98% of oxygen is carried bound to haemoglobin within the RBC. Red blood cells are anucleate cells that do not have organelles and therefore do not participate in protein production nor undergo oxidative metabolism. Energy, in the form of adenosine triphosphate (ATP), is generated within RBCs via anaerobic glycolysis. A molecule in the glycolytic pathway, 1,3-diphosphoglycerate, is converted by RBCs into 2,3-diphosphoglycerate (2,3-DPG). When 2,3-DPG binds to deoxyhaemoglobin, it reduces the affinity of oxygen for haemoglobin, facilitating oxygen release to the tissues.

The partial pressure of oxygen dissolved in the plasma of arterial blood \((\text{PaO}_2)\) is a function of the ability of the lungs to transport oxygen from the atmosphere to the blood. As the \(\text{PaO}_2\) increases, haemoglobin becomes more saturated with oxygen. As dissolved oxygen diffuses out of the blood into the tissues, saturated haemoglobin provides a reservoir of oxygen that prevents a rapid decrease in \(\text{PaO}_2\).
The relationship between \( \text{PaO}_2 \) and haemoglobin saturation (\( \text{SaO}_2 \)) is a sigmoid curve, known as the oxygen-haemoglobin dissociation curve.\(^5\) At the top flat portion of the curve, a large change in \( \text{PaO}_2 \) leads to a small change in \( \text{SaO}_2 \). As the \( \text{PaO}_2 \) declines to reach the steep part of the curve, now a small change in \( \text{PaO}_2 \) can lead to a large change in \( \text{SaO}_2 \).\(^4\) An increase in pH, decrease in temperature, decrease in partial pressure of carbon dioxide (\( \text{PaCO}_2 \)), and/or a decrease in 2,3-DPG causes a shift of the curve to the left.\(^4\) This leftward shift represents an increase in the affinity of haemoglobin for oxygen, with haemoglobin being more saturated at a lower \( \text{PaO}_2 \). A decrease in pH, increase in temperature, increase in \( \text{PaCO}_2 \), and/or an increase in 2,3-DPG causes the oxygen-haemoglobin dissociation curve to shift to the right.\(^4\) This rightward shift represents a decreased affinity of haemoglobin for oxygen, and therefore promotes oxygen release into the tissues.\(^4\)

The shape of RBCs allows for a large surface area to volume ratio which maximises the area for gas exchange, whilst minimising the distance over which gas exchange must occur.\(^3\) An important quality of RBCs is their flexibility, which enables them to traverse capillary beds.\(^6,7\) This flexibility is dependent on viscosity of the cytoplasm, the surface-area-to-volume-ratio and the deformability of the RBC membrane.\(^7\)

Adenosine triphosphate and 2,3-DPG concentrations help to maintain sodium and potassium ionic composition of RBCs which is important for maintenance of deformability.\(^8\) Red blood cells also release ATP during mechanical and hypoxic stress.\(^9\) Binding of ATP to the P2Y receptor on endothelial cells induces the synthesis and release of nitric oxide (NO), a potent vasodilator, thereby regulating local blood flow.\(^9\)
2.1.2 The effect of anaemia on tissue oxygen delivery and oxygen consumption

Tissue oxygen delivery is dependent on cardiac output (CO) and CaO₂. Tissue oxygen consumption (VO₂) is the amount of oxygen utilised by the tissues, and is normally independent of DO₂. In a healthy subject VO₂ is approximately one quarter of DO₂, therefore if DO₂ decreases, the proportion of oxygen extracted by the tissues increases so that VO₂ remains constant. If DO₂ falls below a point where oxygen extraction is unable to increase further to maintain VO₂, VO₂ then becomes dependent on DO₂. Referred to as the critical DO₂, this is the point at which aerobic metabolism can no longer be supported.

Arterial oxygen content is dependent on haemoglobin concentration, SaO₂, and PaO₂. The primary effect of anaemia is a reduction in haemoglobin which reduces CaO₂ and therefore DO₂. Compensation for declining CaO₂ includes an increase in sympathetic outflow and CO. Decreased blood viscosity also results in increased CO. Levels of 2,3-DPG increase within hours of blood loss, shifting the oxyhaemoglobin dissociation curve to the right, facilitating oxygen release to the tissues. When these compensatory mechanisms are unable to maintain DO₂, VO₂ declines and culminates in tissue hypoxia.

2.1.3 The decision to transfuse and the effect of transfusion on tissue oxygen delivery and oxygen consumption

Transfusion of RBCs is not innocuous and can cause adverse effects. The packed cell volume (PCV) at which transfusion is deemed necessary, the transfusion trigger, is dependent not only on the severity of anaemia, but also on history, clinical signs, underlying cause, and clinical judgement. Acute haemorrhage causing a decrease in PCV to 20% or less, equivalent to a haemoglobin concentration of <7.0g/dL, is usually considered an indication for RBC transfusion. However, in acute haemorrhage it can
be difficult to determine if transfusion is necessary, and crystalloid fluid therapy alone may improve CO sufficiently to re-establish DO$_2$. If tissue hypoxia persists despite blood volume expansion with crystalloid fluid resuscitation, then transfusion of RBCs should be considered. Dogs that have chronic blood loss generally tolerate a lower PCV and therefore the transfusion trigger is lower. The target PCV post-transfusion is also dependent on chronicity, and the nature of the underlying disease, but is usually between 25 and 30%. The volume of blood delivered is calculated with reference to the patient’s blood volume, which is 90mls/kg in dogs.

Although transfusion of RBCs is associated with improved DO$_2$, this does not always correlate with improved VO$_2$. In 32 critically-ill people with circulatory shock that were volume resuscitated, all had a haemoglobin concentration <10g/dL and were subsequently transfused with the aim of increasing haemoglobin by 3g/dL. Following transfusion, there was a significant increase in CaO$_2$ and DO$_2$, but no change in VO$_2$ or lactate concentration. A further two studies in mechanically ventilated critically ill people with sepsis also found that blood transfusion did not improve VO$_2$. A more recent study of euvolaemic, anaemic, critically-ill people was unable to demonstrate any beneficial effect of transfusion on DO$_2$.

The ability of transfused RBCs to improve DO$_2$ may be affected by their length of storage. During storage a number of changes occur which can lead to alterations in the shape and function of RBCs, adversely affecting their ability to deliver oxygen to the tissues. A study in rats with isovolaemic haemodilution, from a mean haemoglobin concentration of 10.9g/L to a mean concentration of 30g/L, found a significant increase in VO$_2$ and DO$_2$ in rats transfused with ‘fresh’ blood, stored for three days, when compared with ‘old’ blood, stored for 28 days. A further study in rats in haemorrhagic shock, showed resuscitation with fresh blood resulted in a
return of intestinal microvascular PO$_2$ to baseline, whereas the administration of blood that had been stored for 28 days did not return intestinal microvascular PO$_2$ to baseline.$^{25}$ They also demonstrated a significant reduction in deformability in the older blood products.$^{25}$

2.2. Blood collection, processing and storage

Canine blood products are most commonly sourced from community donor programs, however terminal blood donation does occur in some large referral hospitals. Blood products are aseptically collected and processed into blood components prior to storage. As part of blood processing, whole blood can be passed through a filter to remove leukocytes prior to storage. This leukoreduction reduces the concentration of bioactive mediators within the stored blood product.

2.2.1. Sources of canine blood – community versus terminal donation

In the veterinary profession, there is an increasing utilisation of canine blood transfusions. Large veterinary hospitals and specialist referral centres often maintain a blood donation register to allow for a continuous supply of transfusion products. Another possible avenue for blood collection is to bleed surrendered healthy dogs prior to euthanasia. Each year, over 300 healthy racing greyhounds are retired and then surrendered to the Murdoch University College of Veterinary Medicine for euthanasia. These dogs are not procured or requested of owners and are brought to the university at the convenience of their owners. A very small portion of these greyhounds are able to be re-homed, but most have to be euthanased. The cadavers are then used for teaching purposes or research. To maximise their utility, the Murdoch University Veterinary Hospital has developed a blood collection program approved by the Murdoch University Animal Ethics
Committee. The surrendered greyhounds are anaesthetized and two to four units of blood are collected from each dog prior to euthanasia. These units of blood are then available to transfuse to clinical patients when required. This method of blood collection ensures blood is always available for patients in need, however, there is concern about the quality of the blood products collected. The blood is collected while the dogs are anaesthetised, often mechanically ventilated, and the last one to two bags are collected during the onset of haemorrhagic shock. All of these factors may have implications on the activity of leukocytes, and the concentration of bioactive mediators within the blood units collected.

2.2.2. Canine blood collection

The method of blood collection depends on the type of canine donor being used. For most canine donors, blood is collected whilst they are conscious, with or without mild sedation. The area of the jugular vein is clipped and aseptically prepared. Wearing sterile gloves the clinician inserts the needle from a blood collection system into the jugular vein and blood is collected into a sterile blood collection bag containing anticoagulant preservative. One unit is collected, equivalent to 450mls of whole blood, the line is clamped and the needle removed. The blood donor is generally fed a meal following donation.

Terminal donors are anaesthetised prior to blood collection. A cephalic intravenous catheter is placed to allow the administration of sedation and induction agents. The dogs are then intubated and maintained under gaseous anaesthesia. The right lateral cervical region is clipped of hair and aseptically prepared. A skin incision is made to isolate the carotid artery, and an intravenous catheter is inserted into the artery and capped with a sterile injection port. The needle from the blood collection system is inserted into the capped port, and 450 mL of blood is collected.
into a sterile collection bag containing an anticoagulant solution. At least two units of whole blood are collected from each dog. The dogs are then euthanased via intravenous injection of pentobarbital sodium whilst under anaesthesia. Units of whole blood are then processed as outlined below.

2.2.3. Blood component processing and storage

The ability to process whole blood into its components enables more efficient use of blood products and allows more than one patient to benefit from a single blood donation. Transfusing only the component that is required may also help to reduce adverse side effects. Storage of blood products for extended periods allows for increased availability of blood products when needed.

After collection, fresh whole blood contains RBCs, leukocytes, platelets, and all components of plasma. In dogs, whole blood is collected into bags containing citrate-phosphate-dextrose-adenine -1 (CPDA-1). Citrate is an anti-coagulant; dextrose promotes glycolysis, and therefore helps to maintain 2,3-DPG levels; adenine promotes ATP production, and therefore maintains cell viability and function; and phosphate is a buffer for by-products of RBC metabolism, such as lactate. Each bag is designed to collect 450mls +/- 45mls. If units are not further processed, fresh whole blood should be used within 8 hours of collection. After 4 hours at room temperature platelet activity and coagulation factor activity decline and the risk of bacterial contamination increases. Once refrigerated, stored whole blood is thought to rapidly lose platelet activity and labile clotting factors, including factor V, factor VIII and vWF.

Whole blood units can be processed via centrifugation (5000 x g for five minutes at 4°C) to produce one unit of plasma and one unit of PRBC. Packed red blood cells
contain mostly RBCs, leukocytes, and a small amount of plasma. Fresh frozen plasma (FFP) contains all coagulation factors, including fibrinogen, and a small amount of albumin. Plasma that is not frozen within eight hours of collection or plasma that has been frozen for longer than one year loses labile clotting factors, but is still a good source of vitamin K dependent clotting factors. Plasma can be kept frozen at -20°C as FFP for one year and then from one to five years as frozen plasma. Cryoprecipitate is made by slowly thawing FFP to a slushy consistency, centrifuging (5000 x g for 7 minutes) and removing the supernatant. Cryoprecipitate is the cold insoluble portion of FFP which contains high concentrations of factor VIII, von Willebrand factor (vWF) and fibrinogen. Cryoprecipitate can be frozen for up to one year.

Units of canine whole blood and PRBC are stored at 1-6°C. The shelf life is determined by the number of days at which 75% RBC viability is maintained. In one study, the post transfusion viability of canine PRBC stored in CPDA-1 alone was 90% on day 1, and reduced to 46% on day 40. Additive solutions are able to prolong storage of RBC products for 35 to 42 days. ADSOL® is a common additive solution which contains saline, adenine, dextrose and mannitol. Mannitol is added to help prevent RBC lysis. Canine PRBC collected into CPDA-1 with the addition of ADSOL® had a post transfusion viability of 80% after 37 days of storage, which met the FDA minimum standard for human PRBC.

### 2.2.4. Leukoreduction

Leukoreduction is the process of removing leukocytes from RBCs prior to storage or administration to the patient. There are various techniques that have been used to remove leukocytes, including centrifugation, sedimentation, freeze-thawing, apheresis and filtration. The passage of blood through a leukocyte reduction filter
is the most commonly used method. Up to 99.9% of leukocytes are removed from the blood by adhering to the negatively charged surface of the filter. Pre-storage leukoreduction has been found to be superior to delayed post-storage leukoreduction, as it prevents the accumulation of bioactive mediators within the blood products during storage. A recent study performed leukoreduction of canine PRBC and reported no adverse effect on the viability of canine RBCs that had undergone filtration to achieve leukoreduction.

2.3. Pre-transfusion testing and administration

Prior to transfusion all blood donors and recipients should undergo blood typing and cross-matching to try and prevent acute haemolytic transfusion reactions. In people it is commonplace to pre-medicate patients with anti-pyretics and anti-histamines, however evidence for any benefit is lacking in both people and dogs.

2.3.1. Canine blood groups

The first four recognised canine blood groups were described by Von Dungern and Hirszfeld in 1910. In the 1950s Swisher, Young and co-workers described seven canine blood groups A, B, C, D, E, F and G. They recognised two categories of A antigens, a strongly reactive group they called A1, and a less strongly reacting group they called A2. Between 1972 and 1974 the canine blood group system nomenclature was standardized adopting the dog erythrocyte antigen, or DEA system. The DEA system recognises the following blood groups: DEA 1.1, DEA 1.2, DEA 1.3, DEA 3, DEA 4, DEA 5 and DEA 7. Dog erythrocyte antigen 6 and 8 exist, but there are no typing antisera available. There are other canine erythrocyte antigens that have been reported including the Dal antigen which is found in most of the canine population except for some Dalmations.
2.3.2. Pre-transfusion recipient testing

The ideal definition of a universal donor is negative for DEA 1.1, 1.2, 3, 5 and 7 and positive for DEA 4. The DEA 1.1, 1.2 and 1.3 (A system) antigens are most important clinically as they are the most antigenic and can be associated with acute haemolytic transfusion reactions. Dog erythrocyte antigen 1.1 occurs in approximately 42% of dogs, and DEA 1.2 in 20% of dogs. DEA 1.3 is a newly recognised antigen with a high prevalence in German Shepherd dogs, which has only been detected in Australia. Dog erythrocyte antigens 3, 5 and 7 occur in 6%, 23% and 45% of dogs respectively, whereas the DEA 4 antigen is present in 98% of dogs.

Alloantibodies against DEA antigens 3, 5 and 7 occur naturally in 30%, 10% and 50% of dogs, and can be induced in dogs without naturally occurring alloantibodies, when they are transfused with RBC that have these antigens. Antibody-antigen reactions in these dogs cause a decrease in RBC survival. Alloantibodies against DEA 1.1 are not naturally occurring, but rather are induced antibodies which occur after administration of mismatched RBCs. Dog erythrocyte antigen 1.1 negative dogs that receive DEA 1.1 positive blood develop anti-DEA 1.1 antibodies which with subsequent transfusions will cause rapid agglutination and haemolysis. The production of antibodies following initial transfusion has been reported within 9 to 11 days following transfusion.

Canine donors are typically only blood typed for DEA 1.1 antigen, with an ideal donor being DEA 1.1 negative. Blood typing is performed by adding recipient RBC to known reagent antisera and assessing for haemagglutination. Although antibodies against antigens other than DEA 1.1 are unlikely to cause acute haemolysis, they still have the potential to reduce the lifespan of transfused RBC.
Therefore, cross-matching is ideal prior to all transfusions. A major cross-match assesses compatibility of donor RBC with recipient serum, whilst a minor cross-match assesses compatibility of recipient RBC with donor serum. Red blood cells are incubated with serum and observed for visible agglutination or haemolysis to identify any incompatibilities.41

2.3.3. Premedication

It is common practice in human medicine for transfusion recipients to receive premedication with acetaminophen, and/or diphenhydramine.42-44 These medications are administered prior to transfusion in order to reduce the occurrence of febrile episodes associated with transfusion, otherwise known as febrile non-haemolytic transfusion reactions (FNHTR). A large retrospective study in people over a five year period showed that routine premedication with acetaminophen reduced the overall incidence of FNHTR, which reduced patient morbidity.44 In this study they reported a reduction in FNHTR in patients receiving RBC transfusions from an average of 0.16% to 0.08%. During this study however, leukoreduction was introduced at their institution, and there was no comparison group to isolate the effect of premedication.44 A prospective randomised clinical trial in people assigned blood transfusion recipients to receive either 500mg acetaminophen and 25mg diphenhydramine or a placebo.42 They reported 62 transfusion reactions in 315 patients of which 36 were urticarial and 21 were FNHTR. They found no difference in urticarial reactions between the two groups, but a reduction in FNHTR from 4.4% to 2.2%.42 Their study population however was very small and all blood products in this study were leukoreduced.42 A further retrospective study in children found that FNHTR occurred in 0.95% of 4108 transfusions that were pre-medicated with acetaminophen compared with 0.53%
of 3792 transfusions that were not pre-medicated, and allergic reactions occurred in 0.9% of 4315 transfusions that were pre-medicated with diphenhydramine compared with 0.56% of 3585 transfusions that were not pre-medicated. In conclusion, neither acetaminophen nor diphenhydramine were found to significantly reduce the incidence of FNHTR or allergic transfusion reactions.45

A recent retrospective study in dogs examined 935 transfusions. Of these dogs, 13% (40/312) that were not pre-medicated developed a transfusion reaction, 18% (26/147) that were pre-medicated with steroids developed a transfusion reaction, 14% (45/305) that were pre-medicated with diphenhydramine developed a transfusion reaction, and 19% (33/171) that were pre-medicated with both steroids and diphenhydramine developed a transfusion reaction. Overall they found no significant reduction in transfusion reactions with pre-medication.46

Although acetaminophen and diphenhydramine are considered to have few side effects, prophylactic administration is not without risks. Acetaminophen can cause hepatotoxicity and oxidative stress to RBC.47,48 Diphenhydramine is a H1 receptor antagonist and also has anticholinergic effects which can cause CNS impairment leading to drowsiness and altered mentation.43 The prevention of a febrile response may itself be a risk in that it may delay the diagnosis of life-threatening transfusion reactions such as acute haemolytic reactions and sepsis.47 It is also unknown if premedication is simply preventing a febrile reaction whilst underlying inflammation may still be occurring.47 Finally, routine premedication increases the overall cost of transfusion administration.47
2.3.4. Administration technique

Ideally all transfusion products are warmed to room temperature and are administered via a peripheral intravenous catheter. Initially the transfusion is administered slowly at 0.5-1ml/kg/hr for the first 15 minutes whilst monitoring the patient for any evidence of a transfusion reaction. The transfusion rate is then increased and delivered within four hours. Patient vitals are taken every 15 minutes for the first hour and then every 30 to 60 minutes for the remainder of the transfusion. Blood transfusions may be administered via gravity, a volumetric peristaltic infusion pump, or via a syringe pump. An inline filter is required when administering RBC in order to remove debris and clots which may lead to the formation of emboli. The method of delivery of the transfusion can affect the viability of transfused blood products. A recent study in dogs assessed the lifespan of biotinylated autologous RBC administered to 9 dogs via volumetric peristaltic infusion pump, syringe infusion pump, and via gravity. The study found that RBC administered via volumetric and syringe pumps had a marked decrease in survival compared with RBC delivered via gravity.

2.4. The storage lesion and cytokines

Blood products undergo various changes during storage that can affect RBC viability and function, and they accumulate bioactive mediators such as cytokines. Cytokines are released from cells of the immune system and have pro-inflammatory and anti-inflammatory properties. The presence of cytokines has been demonstrated in both human and canine blood products. Collection of blood from terminal donors in haemorrhagic shock may increase the concentration of cytokines in stored blood products, worsening the storage lesion.
2.4.1. The storage lesion

During storage of RBC-containing products there are a number of physical and metabolic changes that occur. The concentration of 2,3-DPG, and ATP declines, ammonia accumulates, and haemolysis results in the accumulation of free haemoglobin. Leukocytes within the stored blood product also release cytokines and other inflammatory mediators. The end result is a decline in the post transfusion viability of RBCs, and the transfusion of various bioactive mediators that can lead to recipient inflammation and transfusion reactions.

Red blood cells generate ATP via anaerobic glycolysis. Declining levels of glucose throughout storage decrease ATP production. Red blood cells subsequently become crenated, forming echinocytes, spheroechinocytes and ultimately spherocytes. These changes result in increased osmotic fragility, and loss of deformability which can impede microvascular flow. One study monitored RBC deformability and shape throughout storage of human blood products, and found that alterations to RBC shape occurred from the second week of storage, with a progressive decrease in deformability, and an increase in haemolysis and acidosis. In canine PRBC, there was a decline in ATP concentrations at day 10 of storage. The concentration of 2,3-DPG in stored PRBC affects the oxygen affinity of haemoglobin. Declining levels of 2,3-DPG throughout storage leads to a shift in the oxyhaemoglobin dissociation curve to the left, reducing the ability of RBCs to release oxygen to the tissues.

Stored PRBC also accumulate ammonia secondary to the deamination of proteins within these products. Stored canine PRBC were shown to have increased ammonia during storage, increasing from 23 +/- 8 mmol/L on Day 0, to 562 +/-
27mmol/L on day 35. The clinical implication of transfusing higher concentrations of ammonia is unclear, however it would likely be detrimental in patients with liver dysfunction or failure, or in patients receiving massive transfusions.

Haemolysis of RBCs occurs progressively throughout storage which results in the accumulation of free haemoglobin. Post transfusion haemolysis of stored RBCs also releases free haemoglobin. Haemoglobin is then available to scavenge NO, which can lead to changes in vascular function including vasoconstriction and vascular injury in the transfusion recipient. The administration of high concentrations of iron-rich haemoglobin overwhelms the binding capacity of the major physiologic iron carrier, transferrin, and there is an increase in free non-transferrin-bound iron (NTBI). Elevations in circulating iron levels can enhance bacterial pathogenicity. An experimental study in beagles with Staphylococcus aureus pneumonia found that transfusion of older blood (42 days) was associated with greater clinical decompensation and increased mortality, than beagles transfused with fresher blood (7 days). These dogs were bled and then underwent transfusion exchange with commercially available canine leukoreduced PRBC and plasma. The clinical decline was seen with increasing doses of bacterial challenge and was associated with a rapid decline in NTBI and plasma labile iron, suggesting that iron released from RBCs was utilised by bacteria, promoting bacterial growth.

Leukocytes within blood products release bioactive mediators throughout storage into the storage medium. These bioactive mediators can affect the viability of RBCs, and may also have a negative impact on the transfusion recipient. The bioactive mediators released include histamine, lipids, oxygen free radicals and cytokines. The resulting oxidative damage to RBC membranes leads to formation
of methaemoglobin, which is unable to carry oxygen. Alterations to cell membranes (RBCs, leukocytes and platelets) can also lead to the formation of microparticles, which are fragments of the phospholipid membrane. These microparticles have inflammatory and procoagulant properties. The transfusion of these bioactive molecules may incite an inflammatory response in the recipient, and may contribute to the occurrence of transfusion reactions such as febrile non-haemolytic transfusion reactions (FNHTR) and transfusion related acute lung injury (TRALI).

2.4.2. Cytokines associated with transfusion reactions, and their physiological effects

Cytokines are biological mediators that are important in cell to cell communication. They are released from cells of the immune system in response to infectious and inflammatory stimuli, including binding of pathogen associated molecular patterns or damage associated molecular patterns to their pattern recognition receptors. These small proteins travel and bind to nearby cells triggering further biological actions. There are many hundreds of cytokines that interact to create a fine balance between pro-inflammatory and anti-inflammatory states.

There are four major pro-inflammatory cytokines that have been associated with transfusion reactions in people; TNF-α, interleukin-1 (IL-1), interleukin-6 (IL-6), and IL-8. Interleukin-1 cytokines have two structurally similar subgroups, interleukin-1α (IL-1α), and IL-1β. Interleukin-1β is the major subtype of IL-1 that is released from mononuclear phagocytes, whereas IL-1α remains attached to the cell. Tumour necrosis factor-α is released from mononuclear phagocytes as well as many other immune cells, endothelial cells and fibroblasts. Tumour necrosis
factor-α and IL-1β have a pro-inflammatory influence on nearby cells, causing the classical signs of inflammation: redness, heat, swelling and pain. These mediators have many similar biological effects and display synergism. Their effects include enhancing the adhesiveness of vascular endothelial cells to neutrophils, promoting migration and activation of neutrophils, and enhancing the ability of neutrophils and macrophages to kill microbes. The production of IL-6 is triggered by bacterial endotoxins, TNF-α and IL-1. IL-6 is a mediator of the acute phase response promoting inflammation. It also has anti-inflammatory effects, inhibiting some of the effects of IL-1 and TNF-α as well as stimulating the production of interleukin-1 receptor antagonist (IL-1RA) and interleukin-10 (IL-10). Interleukin-1β, TNF-α, and IL-6 are all endogenous pyrogens that stimulate the release of prostaglandin E₂ (PGE₂) from the hypothalamus via humoral or neuronal pathways. Interleukin-8 is a unique type of cytokine called a chemokine, a family of chemotactic molecules, also known as CXCL8. Interleukin-8 is released from many cells including macrophages, neutrophils, endothelial cells and mast cells following inflammatory stimuli. Interleukin-8 induces neutrophils to adhere to endothelial cells and promotes degranulation and stimulation of the respiratory burst. Activation of contractile proteins allows neutrophils to change shape, adhere to the endothelium and migrate into the tissues. Degranulation results in the release of enzymes such as elastase. The respiratory burst is stimulated via activation of NADPH-oxidase leading to the production of potent oxidants which kill bacteria and enhance the activities of lysosomal enzymes. Various inflammatory mediators induce the release of IL-8 from monocytes and macrophages including lipopolysaccharide, IL-1 and TNF-α.

In healthy rabbits, TNF-α given intravenously has been shown to induce endogenous IL-1 production. Administration of IL-1 and TNF-α caused a febrile
response similar to that seen with endogenous pyrogens, and can cause hypotension and haemodynamic changes typical of shock.\textsuperscript{68,73} People with disseminated cancer given intravenous TNF-\(\alpha\) showed similar findings with an increase in temperature and tachycardia, with some people developing hypotension.\textsuperscript{74} Interleukin-8 administration to healthy primates resulted in granulocytopenia followed by granulocytosis with no notable changes to hemodynamic parameters.\textsuperscript{75} Post-mortem histology in these primates showed increased neutrophil margination in the capillaries of the lungs, liver, and spleen, but there were no secondary lesions associated with the presence of these cells.\textsuperscript{75} Although no adverse effects were seen in these healthy primates, it is possible that increasing activation, margination, and degranulation of neutrophils may potentiate organ damage in patients that have systemic disease.

\textbf{2.4.3. Demonstrating the presence of cytokines in transfusion products}

Heddle first theorised that bioactive substances were present in the plasma of platelet concentrates and it was these bioactive substances that were responsible for FNHTR.\textsuperscript{76} The observation that FNHTR occurred more commonly with older transfusion products, and that cytokines were found to accumulate in these products over time, supported this observation.\textsuperscript{76} Heddle proved this theory by transfusing platelet concentrates in two fractions, the cellular component and the supernatant separately. The supernatant was found to be significantly more likely to cause a reaction when compared with the cellular component. Heddle then went on to characterise these bioactive substances as cytokines, IL-1\(\beta\) and IL-6, and demonstrated that these cytokines accumulated during storage of platelet products.\textsuperscript{77} A similar study found the same association between FNHTR and storage
time when transfusing platelet products, and demonstrated the accumulation of IL-6 and TNF-α during storage.⁷⁸

Many studies have since measured cytokine concentrations in human stored RBC products, including whole blood, PRBC, buffy coat reduced PRBC and bacterially contaminated PRBC.⁵³-⁵⁶,⁷⁹,⁸⁰ Although study results varied, the overall trend was that IL-1β, IL-8 and TNF-α accumulated in blood products during storage, and IL-6 concentrations either did not increase or were not detectable throughout storage. A number of these studies measured cytokine concentrations in leukoreduced units and found that leukoreduction prevented the accumulation of these inflammatory cytokines.⁵³,⁵⁴,⁷⁹,⁸⁰

There is only one study which confirms the presence of cytokines in stored canine PRBC.⁸¹ This study measured IL-1β, IL-8, IL-10 and TNF-α in NLR and LR canine PRBC, weekly for 5 weeks of storage. They found that IL-8 significantly accumulated in NLR units over time.

2.4.4. The development of haemorrhagic shock in blood donors may increase cytokine production

During haemorrhagic shock the initial life-sustaining mechanisms that occur include the baroreceptor reflex, a profound sympathetic response, release of angiotensin and vasopressin, and the redistribution of fluid from the extra-vascular to the intra-vascular space.¹¹ These mechanisms aim to maintain cardiac output and blood pressure to vital organs such as the heart and brain.¹¹ As a result, decreased perfusion to other tissues such as the gastrointestinal tract can lead to mucosal sloughing, translocation of bacteria and release of inflammatory mediators.¹¹
Cellular hypoxia ultimately leads to cellular dysfunction and death causing multiple organ dysfunction.\textsuperscript{11}  

An experimental study in rats subjected to trauma and haemorrhage found that TNF-\(\alpha\) levels were significantly elevated 45 minutes into haemorrhage.\textsuperscript{82} The authors felt that the elevation in TNF-\(\alpha\) was mostly due to haemorrhage but could not rule out an effect of trauma. Another study in mice by the same authors found that haemorrhage alone, without trauma, resulted in significantly elevated levels of TNF-\(\alpha\) at 30 minutes into hemorrhage.\textsuperscript{83} A recent study in anaesthetised pigs with induced liver injury and haemorrhage found that levels of IL-6, IL-8 and TNF-\(\alpha\) were all significantly elevated from baseline to one hour and from baseline to two hours.\textsuperscript{84} A study in humans found that patients with haemorrhagic shock secondary to ruptured abdominal aortic aneurysm had significantly elevated TNF-\(\alpha\) and IL-1\(\beta\) at hospital admission.\textsuperscript{85} This increase was significantly greater than in trauma patients. These studies suggest that there is a rapid increase in inflammatory mediators following haemorrhage. Therefore, blood collected from anaesthetised dogs with haemorrhagic shock may contain more bioactive mediators than blood collected from healthy conscious dogs.

2.5. \textbf{Adverse effects of blood transfusion}

Blood transfusions can be life-saving, however many adverse effects can occur. Transfusions in people and dogs are associated with increased morbidity and mortality.\textsuperscript{86,87} The risk of morbidity and mortality also increases with storage time, likely due to the accumulation of bioactive mediators.\textsuperscript{20,22,88} Reactions associated with the transfusion of bioactive mediators include acute inflammation, FNHTR and TRALI.\textsuperscript{6,7,57,66,89}
2.5.1. Mortality and transfusion

Blood transfusion is an independent risk factor for increased morbidity and mortality in people. A large scale study called Anaemia and Blood Transfusion in Critically Ill Patients, or the ABC study, evaluated 3534 critically-ill people from 146 ICUs.\textsuperscript{86} The overall transfusion rate was 41.6%. Transfusion was associated with longer ICU stays with a mean ICU length of stay of 7.2 days compared with 2.6 days for non-transfused patients. Mortality in transfused patients was higher than non-transfused patients, 29% vs. 14.9%. The overall mean pre-transfusion haemoglobin level was 8.4g/dL, and transfusions were given to less than 30% of patients with a haemoglobin concentration greater than 9g/dL. The overall findings were greater mortality and decreased organ function in transfused patients. A similar study called Anaemia and Blood Transfusion in the critically ill, or the CRIT study, had comparable findings.\textsuperscript{87} They enrolled 4,892 people in 284 ICUs, and reported that 44% of ICU patients received at least one transfusion. The mean pre-transfusion haemoglobin was 8.6g/dL. They found that the length of hospitalisation and mortality was associated with the number of transfusions received. These studies used illness severity scoring to account for the likelihood that people who are more critically ill are more likely to receive blood transfusions and have a poorer outcome.

To illustrate the effects of transfusion independent of illness severity, a large number of studies have randomly assigned patients to restrictive versus liberal transfusion strategies. These studies have consistently shown that a restrictive transfusion strategy is associated with lower mortality and fewer adverse effects.\textsuperscript{90-92} A large multicentre, randomised, controlled clinical trial of transfusion requirements in critical care, the TRICC study, compared a restrictive RBC transfusion strategy with a liberal RBC transfusion strategy.\textsuperscript{90} The restrictive group
were transfused when their haemoglobin concentration dropped below 7.0g/dL, whereas the liberal group received a transfusion when their haemoglobin dropped below 10.0g/dL. They found no significant difference in the primary outcome of mortality between the two groups, 18.7% for the restrictive group, and 23.3% for the liberal group. A sub-group analysis found a significant reduction in mortality in the restrictive group of patients that were less than 55 years of age (5.7% vs. 13.0%) and those with an Acute Physiology and Chronic Health Evaluation II score of 20 or less (8.7% vs. 16.1%). Overall, patients in the restrictive group received 50% fewer transfusions than patients in the liberal group. There are several other studies in people investigating restrictive versus liberal transfusion strategies in many patient categories including paediatric trauma patients, patients with gastrointestinal bleeding, and patients with acute myocardial infarction. These studies report increased mortality with liberal transfusion strategies. The large volume of literature in recent years has since been further analysed in multiple meta-analysis and review articles. The vast majority of evidence suggests that restrictive transfusion strategies are safe in the majority of clinical settings, reduce the number of RBC transfusions, reduce adverse effects associated with transfusion, and are more cost-effective. Some of these studies reported lower mortality and reduced hospitalisation with restrictive transfusion, whilst others showed no difference between restrictive and liberal transfusion groups. The variability in these findings may be associated with the patient population studied, as some studies looked specifically at patients with trauma or perioperative patients, whilst other studies looked at the hospital population as a whole. One meta-analysis evaluated 3469 critically ill patients in 10 trials, and found no difference between liberal and restrictive transfusion strategies, however when they evaluated 7552 surgical patients in 17 trials, they actually found a
significant reduction in mortality with a liberal transfusion strategy. A recent meta-analysis examined 31 trials incorporating 9813 people found that restrictive transfusion strategies reduced the number of RBC transfusions with no significant difference in mortality or morbidity when compared with liberal transfusion strategies. This study incorporated perioperative patients, patients with acute blood loss, critical care patients and trauma patients. A meta-analysis incorporating 2364 critically ill patients from three clinical trials showed that a restrictive transfusion strategy reduced mortality, re-bleeding, acute coronary syndrome, pulmonary oedema and bacterial infections, when compared with a liberal transfusion strategy, whilst a further meta-analysis of 3469 critically ill patients from ten trials found no difference in mortality. Therefore the benefit of restrictive vs. liberal transfusion strategies differs depending on the patient population studied. It can be concluded that in the general hospital population, a restrictive transfusion approach is unlikely to do harm and may in fact improve outcome.

There are few studies in dogs that assess the relationship between blood transfusion, morbidity, and mortality. A recent retrospective study examined 86 dogs that underwent adrenalectomy and found that intraoperative transfusion was associated with poor short-term survival. A further retrospective study evaluated 542 dogs undergoing splenectomy for splenic masses. In this study 44% of dogs received transfusion and these dogs were more likely to die or be euthanased. Evaluation of 83 dogs with haemoperitoneum, of which 90% were the result of splenic bleeding, found that massive transfusion was a negative prognostic indicator. In dogs requiring blood transfusions following trauma, the necessity for blood transfusion was associated with a lower survival rate. A retrospective study of 211 dogs that required transfusion for various causes, found that dogs that
required more transfusions were more likely to die.\textsuperscript{103} Unfortunately, with nearly all of these studies, there was no comparison of disease severity between dogs that were transfused and dogs that were not. It is very likely that dogs with more severe injury or disease were more likely to require transfusion, and therefore had a lower chance of survival. In support of this, dogs in the splenectomy study that required transfusion had a higher illness severity score.\textsuperscript{100} Contrary to these findings, a retrospective study of 110 dogs with immune mediated haemolytic anaemia, a common condition in dogs requiring transfusion, found no association between administration of a transfusion and survival to discharge.\textsuperscript{104}

2.5.2. Fresh blood versus stored blood

The accumulation of bioactive mediators in blood products occurs progressively over time. Therefore, transfusion products that have been stored for longer periods have higher concentrations of these mediators, and will have a higher likelihood of contributing to patient morbidity and mortality.\textsuperscript{20,22,88} The clinical effects of transfusing older blood products compared with fresh blood products, has been extensively studied in humans. These studies have variable results and further studies are currently underway.

Paediatric cardiac surgery patients receiving RBC transfusions stored for more than four days required more RBCs and FFP transfusions,\textsuperscript{105} and in another study were more likely to have postoperative morbidity including pulmonary complications, acute renal failure and infections.\textsuperscript{106} In adult cardiac surgery patients, perioperative transfusion of blood stored for >14 days, was associated with a greater risk of intubation beyond 72 hours, renal failure, sepsis and mortality.\textsuperscript{107} This study retrospectively evaluated 2872 patients who received blood <14 days old, and 3130 patients that received blood >14 days old. A limitation of this study was that the
incidence of abnormal left ventricular function, mitral valve regurgitation and peripheral vascular disease were all significantly higher among patients who received blood that was >14 days old. Another study evaluating 897 cardiac patients did not find a relationship between ICU stay, mechanical ventilation time, perioperative rates of infarction, mediastinitis, or sepsis, and the age of transfused RBCs.\textsuperscript{108} They did however find that patients that received blood that was stored longer than 28 days, were more likely to develop nosocomial pneumonia. A large multi-centre randomised trial of patients undergoing cardiac surgery, the RECESS study, enrolled 538 patients that received RBCs stored for less than ten days, and 560 patients who received RBCs stored for greater than 21 days.\textsuperscript{109} The number of transfusions administered did not differ between groups. They found that the duration of RBC storage was not associated with the development of multiple organ dysfunction, or 7 day or 21 day mortality.

In critically ill children it was found that receiving stored blood ≥14 days increased the occurrence of multiple organ failure and led to a longer stay in the ICU.\textsuperscript{110} A study of 63 trauma patients found that patients who developed multiple organ dysfunction were significantly more likely to have received older blood products.\textsuperscript{111} A further study evaluating 31 people with severe sepsis found no difference between survivors and non-survivors with respect to the number of units transfused, but did find that the age of PRBC transfused correlated with mortality.\textsuperscript{112}

A large multicentre, randomised, blinded trial of critically ill patients enrolled 1211 patients to receive fresh RBCs stored less than eight days, and 1219 patients to receive standard issue RBCs, i.e. the oldest compatible units available.\textsuperscript{113} Units were stored for a mean of 6.1 +/- 4.9 days in the fresh-blood group, and 22 +/- 8.4
days in the standard-blood group. The study did not find a significant difference in 90-day mortality between the two groups.\textsuperscript{113} A further large multi-centre, double-blinded study of critically-ill intensive care patients, TRANSFUSE, is currently in progress and aims to determine if the administration of the freshest available compatible allogenic RBCs will reduce 90 day mortality.\textsuperscript{114}

A recent meta-analysis reviewed 6 randomised controlled trials (RCT) and 31 observational studies evaluating the association between the age of RBC products and survival.\textsuperscript{115} The RCT evaluated the use of fresh blood (one to ten days old) compared with blood stored for an average of 14-21 days, and found no difference in survival. The observational studies examined used significantly older blood products than the RCT, and the authors did find an increasing risk of death with increasing age of RBCs. Another meta-analysis evaluated observational studies and found that in a subset of ICU, cardiac surgery and trauma patients, prolonged storage appeared to be associated with increased mortality.\textsuperscript{116} An earlier meta-analysis evaluated 3 RCT and 18 observational studies, and found that mortality was higher in patients transfused with older blood.\textsuperscript{117} They concluded however that 97 patients would have to be transfused with fresh blood in order to save one life. These studies show an overall trend towards worsening outcomes for patients that receive older blood products. It appears that certain patient populations, such as those undergoing cardiac surgery, and patients with sepsis or trauma, may benefit from the use of fresh blood products.

There is only one retrospective study investigating the clinical effect of transfusing older blood products in dogs.\textsuperscript{103} This study reviewed 211 dogs receiving blood transfusions and did not find any association between the age of PRBC and transfusion associated complications. In this study, dogs were evaluated in two
groups, those that received PRBC stored <14 days to those stored >14 days. The
study did not find any association between the age of PRBC and survival, but was
underpowered to detect this finding.\textsuperscript{103} In an experimental beagle study, it was
found that transfusion of older blood (42 days) was associated with worse outcome
in dogs with induced \textit{Staphylococcus aureus} pneumonia.\textsuperscript{63} A later study by the
same group investigated washing RBCs prior to transfusion, in a similar cohort of
beagles induced with \textit{Staphylococcus aureus} pneumonia.\textsuperscript{118} They found that
washing of older RBCs reduced plasma iron concentration and improved outcome,
whilst washing of fresh RBCs increased cell-free haemoglobin and worsened
outcome.

\subsection{Transfusion induces acute inflammation}

In people, blood transfusions have been shown to cause acute inflammation. In 76
critically ill non-septic patients, 76\% developed leukocytosis post transfusion,\textsuperscript{57}
which was positively correlated with increasing IL-8 concentrations in the stored
RBC products.\textsuperscript{57} Similar findings were reported in 50 critically ill people with 90\% of
these patients developing a post-transfusion leukocytosis\textsuperscript{66}.

One study evaluated nine infected and 11 non-infected people, for 24-48 hours
following surgery.\textsuperscript{119} In the non-infected patients there was a correlation between
the number of units of PRBC transfused and the plasma IL-6 concentration.

Another study evaluating cardiac surgical patients found similar findings with IL-6
concentration increasing in patients in association with the transfusion of PRBC.\textsuperscript{120}
In this study IL-6 could not be detected in the PRBC transfused, suggesting that IL-6
release was stimulated endogenously post transfusion.\textsuperscript{120} An in vitro study
demonstrated that PRBC supernatant separated from PRBC stored for 42 days was
able to stimulate cytokine release from washed human neutrophils.\textsuperscript{121} The PRBC
supernatant was incubated with the washed neutrophils for 24 hours and IL-β, IL-8, TNF-α and secretory phospholipase A₂ (sPLA₂) were measured. The addition of PRBC supernatant to the neutrophil suspension resulted in a marked increase in IL-8 and sPLA₂.

Similarly, transfusion administration has been linked to the development of an inflammatory response in dogs. In one study, thirteen dogs were randomised to receive LR or NLR autologous PRBC that had been stored for 21 days. The leukocyte count, plasma fibrinogen and C-reactive protein (CRP) concentrations were measured pre and post-transfusion. The authors found that all of these parameters significantly increased from baseline after transfusion of autologous NLR stored PRBC. These findings support the concept that administering PRBC induces significant inflammation in the transfusion recipient. They concluded that the inflammatory response was most likely due to the accumulation of bioactive mediators in the transfusion products during storage, as dogs that received LR stored PRBC did not show similar elevations. Another study, in healthy dogs, found that transfusion of blood stored for 28 days induced production of monocyte chemoattractant protein-1, and an increased neutrophil count. These studies identify the presence of bioactive mediators within transfusion products which may trigger the endogenous release of inflammatory cytokines, and may be responsible for immunologic transfusion reactions such as FNHTR and TRALI.

2.5.4. Febrile non-haemolytic transfusion reactions

A febrile response defined as a rise in temperature of at least 1°C, during or shortly after blood transfusion, is known as an FNHTR. In humans, the frequency of FNHTR is reported to occur in 1-6.8% of RBC transfusions and 18-37.5% of platelet transfusions. The increased frequency seen with platelet transfusion is thought
to be due to room temperature storage of platelet products allowing for increased metabolic activity.\textsuperscript{54,76} Patients suffering from FNHTR experience fever, chills, and rigors which can cause significant discomfort.\textsuperscript{76,125}

There are two mechanisms by which FNHTR are thought to occur. The first mechanism is associated with the production of leukocyte antibodies by the recipient, which react with donor leukocytes to cause the release of endogenous cytokines.\textsuperscript{38,123,124,126-128} A second mechanism involves the presence of inflammatory cytokines within the stored blood product\textsuperscript{54,55,123,129}. During storage, leukocytes continue to produce inflammatory cytokines via normal metabolic processes and also release cytokines and other bioactive mediators during apoptosis.\textsuperscript{126} These inflammatory cytokines include IL-1\(\beta\), IL-6, IL-8, TNF-\(\alpha\), and other inflammatory mediators such as histamine, serotonin and acid phosphatase.\textsuperscript{54,79,123,127,129,130} These cytokines cause pyrexia by inducing the synthesis of PGE\(_2\) in the hypothalamus, resulting in elevation of the thermostatic set point.\textsuperscript{68} In 60 people experiencing FNHTR, there was a significant increase in IL-6 and IL-8 post transfusion.\textsuperscript{127}

The frequency of FNHTR in veterinary patients is not well reported. A recent retrospective study of 211 PRBC transfusions in dogs reported a FNHTR incidence of 24\%.\textsuperscript{103} The criterion to diagnose an FNHTR in this study was a rectal temperature greater than 39°C, during or after PRBC transfusion. By using an absolute temperature instead of a change in temperature, this study may have over-reported the occurrence of febrile reactions, as some of the dogs may have had an elevated temperature prior to transfusion commencement. Determining the frequency of FNHTR in animals can be difficult as not all transfusion recipients have their temperatures closely monitored. It has also been demonstrated that dogs can develop an inflammatory response after transfusion that is not always
associated with a febrile response. Similarly in people, a study of 41 patients receiving 117 RBC transfusions and 65 platelet transfusions, found that 28 patients had reactions associated with chills, rigors and discomfort, consistent with FNHTR, but only three of these people were febrile.

2.5.5. Transfusion related acute lung injury

Transfusion related acute lung injury is widely recognised in people following blood transfusion. The syndrome is defined by development of acute lung injury (ALI) within 6 hours of transfusion that is not associated with an alternative risk factor for ALI. Acute lung injury is defined as an acute onset of hypoxaemia with bilateral infiltrates on radiographs and no evidence of left atrial hypertension. Transfusion related acute lung injury is thought to be caused by the presence of antibodies within the donor plasma that react with leukocytes in the recipient. Alternatively, administration of lipids and cytokines within the transfusion product may directly activate leukocytes within the pulmonary capillaries. These activated leukocytes release inflammatory mediators, increasing capillary permeability and ultimately causing pulmonary oedema. The incidence of TRALI in people varies with the blood product administered and is reported to be between 0.04% and 0.16% of transfused patients. Although it has a low incidence, TRALI is responsible for up to 16.3% of deaths associated with transfusion.

The incidence of TRALI in dogs is not known and there is little published on this syndrome in dogs. There is one prospective observational study that assessed 54 dogs that received transfusions for various clinical conditions. The incidence of TRALI in this study was 3.7%. The incidence of TRALI in dogs and people is likely
under-reported, as clinical signs seen with TRALI are often attributed to the underlying disease process.

2.6. **Leukoreduction prevents transfusion reactions**

Leukoreduction prevents the accumulation of bioactive mediators in blood transfusion products and therefore reduces the likelihood of transfusion reactions.\(^1,34,52,65,129,134\)

Leukoreduction has been shown to reduce bioactive substances in human transfusion products including myeloperoxidase, eosinophil cationic protein, histamine and plasminogen activator inhibitor-1.\(^34,129\) In dogs, leukoreduction has been found to prevent the accumulation of IL-8, inflammatory microparticles, and vascular endothelial growth factor.\(^52,65,134\) The clinical benefit of leukoreduction has been well reported in people with a reduction in FNHTR,\(^123,124,135-137\) reduced mortality,\(^136,138\) decreased incidence of TRALI,\(^139\) decreased incidence of post-operative infections\(^140\) and a reduction in the transmission of infectious diseases such as cytomegalovirus.\(^141\)

Since the observed benefit of leukoreduction, many countries have instituted universal leukoreduction schemes, and have reported a reduction in transfusion reactions. A large retrospective study in humans found a significant reduction in the incidence of FNHTR when universal leukoreduction was implemented.\(^124\) The rate of FNHTR prior to leukoreduction was 0.4%, and following the introduction of universal leukoreduction, dropped to 0.2%. Of patients that had multiple transfusions with NLR blood, 24% experienced FNHTR, compared with 10.3% of patients transfused with LR blood. A large scale universal leukoreduction study in Canada enrolled 14 786 surgical patients and found a significant reduction in FNHTR.\(^136\) The proportion of febrile episodes decreased from 24.7% to 22.5% following universal leukoreduction, with a concurrent decrease in the use of antibiotics. They also found a significant reduction in mortality, from 7.03% to 6.19%.

Three other studies that evaluated the effect of universal leukoreduction consistently
found a significant reduction in the rate of FNHTR. A further retrospective study did not find a significant reduction in FNHTR following the introduction of universal leukoreduction, however in this study, selective bedside leukoreduction was performed prior to the introduction of universal leukoreduction.

There are currently no prospective clinical studies in dogs that have investigated the use of LR blood. A study in dogs demonstrated an inflammatory response in healthy dogs receiving stored NLR PRBC units, indicated by elevated leukocyte counts, CRP and fibrinogen levels. In this study, a second group of dogs were given LR stored PRBC units, and no inflammatory response was reported.

Leukoreduction increases the cost and preparation time of producing PRBC. However, in light of preventing accumulation of bioactive mediators, and a likely reduction in patient morbidity and mortality, leukoreduction may be found to be very cost effective. Further large scale clinical studies are needed to determine the role of bioactive mediators on transfusion morbidity and mortality in dogs, and the effects of leukoreduction.
2.7 References


3. Chapter 3: Leukoreduction Prevents Accumulation of Interleukin-8 in Canine Packed Red Blood Cells During Storage

3.1 Abstract

Objective - The aim of this study was to measure and report changes in interleukin-8 (IL-8), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) concentrations in canine stored packed red blood cells (PRBC) over time and assess the effect of leukoreduction on these cytokine concentrations.

Animals - Twelve anesthetized healthy greyhounds

Procedures - One unit of blood was collected from each dog and processed into PRBC. Half of each PRBC unit was passed through a leukoreduction filter to produce a leukoreduced unit (LR) and the remaining blood set aside as a non-leukoreduced unit (NLR). All units had a complete blood count performed on day 0 and were then stored at 2 to 6°C. Samples were collected from the units at days 0, 10, 20, 30 and 37, which were centrifuged and the supernatant stored at -80°C. Canine TNF-α and IL-8 were assessed using a multiplexed genomic and proteomic biomarker analyzer and canine IL-1β was measured by enzyme-linked immunosorbent assay.

Results - Leukoreduction resulted in a 99.9% reduction of leukocytes in all LR units. Both TNF-α and IL-1β concentrations were not significantly different between LR and NLR units, and did not change significantly over time. Interleukin-8 concentration was significantly higher in NLR units than LR units on all days (P < 0.002). The IL-8 concentration in LR units did not increase during storage.

Conclusions and Clinical Relevance - This study showed that leukoreduction was effective for the removal of leukocytes from canine PRBC, which prevented accumulation of IL-8 during storage. Leukoreduction may therefore reduce cytokine associated complications of transfusion.
3.2 Introduction

Transfusion of blood products can result in recipient inflammation in people and dogs. The effect of inducing inflammation after blood transfusion in critically ill patients is unknown but may increase morbidity and mortality. Inflammation associated with transfusion results, in part, from the accumulation of cytokines in stored blood products. Interleukin-1β (IL-1β), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) are cytokines that increase in human whole blood and packed red blood cells (PRBCs) during storage.

Leukoreduction is the practice of filtering blood products to remove white blood cells. Leukoreduction prior to storage is effective in removing leukocytes from human PRBCs and preventing cytokine accumulation during storage. In both people and dogs, recipients of non-leukoreduced (NLR) PRBCs have evidence of enhanced inflammation compared to those that receive leukoreduced (LR) PRBCs. Also, use of LR blood products has been associated with lower mortality compared to NLR blood products in people.

One study evaluating canine stored PRBCs for the presence of IL-1β, IL-8, TNF-α, and interleukin-10, reported only IL-8 concentrations accumulating over time in NLR PRBCs and leukoreduction prevented the accumulation of this cytokine. This study had reported a large variation in concentrations of the other cytokines, and consequently was underpowered to find a difference in IL-1β, TNF-α, and IL-10 between stored LR and NLR units.

The aim of our study was to measure IL-1β, IL-8, and TNF-α concentrations in canine PRBCs during storage and to assess the effect of pre-storage leukoreduction on cytokine concentrations. A paired design was used to reduce variation, with each PRBC unit divided into two aliquots: one LR and one NLR. We hypothesized that canine NLR PRBCs would
have significantly higher concentrations of IL-1β, TNF-α, and IL-8 after 37 days of storage compared to canine LR PRBCs.

3.3 Materials and Methods

Animals- Twelve healthy retired racing greyhounds that had been donated to the university were used for this study. There were 7 male and 5 female greyhounds aged between 17 months and 3 years of age. They were determined to be healthy based on a physical examination performed by a staff veterinarian. All dogs had a normal complete blood count and were blood typed DEA 1.1 negative. The study protocol was approved by the Institutional Animal Ethics Committee (R2564/13).

Blood collection and processing- Each greyhound was sedated with intramuscular methadone (0.3mg/kg). A 21 gauge intravenous catheter was placed in the cephalic vein and anesthesia was induced with intravenous alfaxalone (2.3 to 3mg/kg) and maintained under anesthesia with isoflurane (0.5 to 2.5 %) in 100% oxygen via an endotracheal tube. Anesthetic depth, heart rate, respiratory rate, and blood pressure were monitored.

Manual positive pressure ventilation was performed during periods of apnea.

The right lateral cervical region was clipped and aseptically prepared. The skin was incised with a scalpel. The carotid artery was isolated and a 14 gauge intravenous catheter was inserted into the artery and capped with a sterile injection port. The needle from the blood collection system was inserted into the capped port and 450 mL of blood was collected into the bag containing citrate, phosphate, dextrose, and adenine formula 1 (CPDA-1) anticoagulant. In total, three units of blood were collected from each dog prior to euthanasia. The blood was then centrifuged and a plasma extractor was used to transfer the plasma into a satellite bag. An integrally-attached red blood cell preservative, additive solution 3 (AS-3), was then added to the unit of PRBCs. Half of each unit was then passed
through a leukoreduction filter\(^d\) into a separate storage bag\(^c\) to produce a LR unit and the other half was passed unfiltered into a separate storage bag\(^c\) to produce a NLR unit. All 24 units (12 LR and 12 NLR) were stored at 2 to 6°C and were mixed by inversion every 24 to 48 hours.

Bag Sampling- A blood bag sampling spike\(^d\) was aseptically inserted into the PRBC bag port for sample collection during the study. A 12 mL blood sample was aseptically collected from each bag immediately prior to storage (day 0). A complete blood count was performed on 2 mL using an automated hematology analyzer\(^e\) and the remaining sample was centrifuged and the supernatant stored at -80°C. Further 10 mL samples were collected aseptically from each bag on days 10, 20, 30, and 3716. All samples were centrifuged and the supernatant stored at -80°C for later cytokine analysis.

Cultures- At completion of the project, a 10 mL sample from each PRBC unit was added to a blood culture medium and sent to an outside microbiology laboratory for aerobic and anaerobic bacterial culture.

Cytokine Assessment- Both TNF-α and IL-8 were assessed using a canine cytokine magnetic bead panel\(^f\) in our in-house multiplexed genomic and proteomic biomarker analyzer\(^g\). Canine IL-1β was measured using an in-house enzyme-linked immunosorbent assay\(^h\). These tests were performed according to the manufacturers’ guidelines.

Statistical Analysis- Statistical analysis was performed with commercially available software.\(^i\) Prior to data collection, a power calculation was performed to estimate adequate sample size for the study. Sample size estimations were performed with alpha set at 0.05 and power set at 0.80 (beta = 0.20) to test the hypothesized effect between stored NLR PRBCs and stored LR PRBCs. Since it was expected that the LR PRBCs would have a negligible concentration, a paired sample size of 4 was adequate to prove a response was
different from zero. To demonstrate a significant difference over time, expected effect sizes (difference in means/SD) of 0.25 to 1.0 were estimated from previous work in people. Paired sample sizes of 128 to 10 would be required to detect these, respectively. A sample size of 12 was chosen to detect an effect size of at least 1.0, which was estimated as the expected difference between day 0 and day 37.

The bag weights and hematocrit of each unit were normally distributed, verified by failure to reject the null hypothesis of normality (Shapiro-Wilk test, \( P > 0.05 \)), and were analysed using a paired t-test \( (P < 0.05) \). The concentrations of IL-1\( \beta \), IL-8, and TNF-\( \alpha \) were positively skewed and failed to follow a normal distribution, verified by rejection of the null hypothesis of normality using the Shapiro-Wilk test \( (P < 0.05) \). The concentrations are presented as medians (interquartile range). A square root transformation of the IL-1\( \beta \), IL-8, and TNF-\( \alpha \) concentration created a normally distributed response, verified by failure to reject the null hypothesis of normality using the Shapiro-Wilk test \( (P > 0.05) \). The concentration of IL-1\( \beta \), IL-8, and TNF-\( \alpha \) were each analysed using a mixed effect model, evaluating the fixed effects of blood units (LR, NLR) and time (day 0, 10, 20, 37) accounting for the random variance of dogs across paired blood units and the repeated time points. Where there was significant interaction of blood units and time \( (at P < 0.05) \), selected comparisons were made across blood units and time points against a Scheffe-adjusted \( P<0.05 \).

### 3.4 Results

The mean (±SD) weight of each whole unit of PRBCs was 400g (±18g). The mean (±SD) weight of each LR unit was 180g (±6g), and each NLR unit was 173g (±13g). There was no significant difference in bag weights between the LR and NLR units \( (P = 0.981) \). The mean (±SD) weight of blood lost to the leukoreduction filter was 47g (±5g). The hematocrit of LR units ranged between 0.61 and 0.71, the hematocrit of NLR units ranged between 0.63 and
There was no significant difference in hematocrit between the LR and NLR units \((P = 0.336)\). Leukocyte counts in NLR units ranged between \(3.1 \times 10^9 \) cells/L and \(10.7 \times 10^9 \) cells/L. Leukoreduction was effective as our hematology analyzer showed values below the detectable limit for all leukocyte counts post reduction. This translates to a leukocyte count of \(<0.00002 \times 10^9 \) cells/L, which equates to a leukocyte reduction of >99.9% in all LR units. Platelet counts in NLR units ranged between \(174 \times 10^9 /L\) and \(336 \times 10^9 /L\), and in LR units ranged between \(2 \times 10^9 /L\) and \(8 \times 10^9 /L\), a mean reduction of >98%. Bacterial cultures showed no growth for all units.

The IL-1β concentrations were not significantly different between LR and NLR units, and did not change significantly over time \((P = 0.3508)\) (Table 1).

**Table 1**—Median (interquartile range) cytokine concentrations (pg/mL) at predetermined time points in leukoreduced and nonleukoreduced PRBC units created from whole blood samples collected from 12 anesthetized healthy Greyhounds.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukoreduced</td>
<td>17 (0–25)</td>
<td>17 (2–30)</td>
<td>15 (4–30)</td>
<td>17 (3–37)</td>
<td>13 (5–35)</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukoreduced</td>
<td>316 (222–425)*</td>
<td>353 (267–436)*</td>
<td>436 (338–512)*</td>
<td>452 (390–568)*</td>
<td>472 (361–597)*</td>
<td></td>
</tr>
<tr>
<td>Nonleukoreduced</td>
<td>1,606 (1,149–2,314)†</td>
<td>4,005 (2,334–5,693)†</td>
<td>4,838 (2,778–6,060)†</td>
<td>5,367 (3,003–7,167)†</td>
<td>4,346 (3,369–5,280)†</td>
<td></td>
</tr>
</tbody>
</table>

*Within a column for a given cytokine, median concentration was significantly \((P < 0.001)\) higher for nonleukoreduced units than for leukoreduced units.

†Within a row, median cytokine concentration is significantly (Scheffe adjusted \(P \leq 0.05)\) different from that on day 0.
The TNF-α concentrations were less than the minimum detectable value (6.1 pg/mL) in all NLR and LR units at all time points. The IL-8 concentrations were significantly lower in the NLR units on day 0 than on any of the other days (P < 0.001). Concentrations of IL-8 in the NLR units were significantly higher than concentrations in the LR units at all time points (P < 0.001), and IL-8 concentrations did not change significantly during storage in LR units (Table 1).

### 3.5 Discussion

Our study showed IL-8 accumulated in NLR canine PRBCs during storage, whereas IL-1β and TNF-α did not. Additionally, IL-8 was significantly lower in LR units compared with NLR units on all days. These findings are in accordance with a recent study that measured IL-1β, IL-8, and TNF-α in canine PRBCs.15

Interleukin-8 is one of a family of chemotactic molecules, the chemokines.17 It is released from many cells including macrophages, neutrophils, endothelial cells, and mast cells following inflammatory stimuli.18,19 Interleukin-8 is the primary activator of neutrophils, inducing adherence to endothelial cells, and promoting degranulation and respiratory burst.9,17,18,20 It is unclear if transfusing blood products with high concentrations of IL-8 is associated with increased patient morbidity. Effects of IL-8 administration to healthy primates were minor, including initial granulocytopenia followed by granulocytosis with no notable changes to hemodynamic parameters.21 Post-mortem histology in these primates showed increased neutrophil margination in the capillaries of the lungs, liver, and spleen, but there were no secondary lesions associated with the presence of these cells.21 Although no adverse effects were seen in these healthy primates, it is possible that increasing activation, margination, and degranulation of neutrophils may potentiate organ damage in patients that have systemic disease.
Our study reported a markedly higher concentration of IL-8 in NLR PRBCs than has been reported in either stored human or canine transfusion products.\textsuperscript{9-11,15,22} On day 37, all of the NLR PRBC units had IL-8 concentrations exceeding 3300 pg/mL, with the maximum concentration exceeding 14000 pg/mL. A study in people reported a much lower mean IL-8 concentration of 745 ± 710 pg/mL in PRBCs after four weeks of storage.\textsuperscript{1} The maximum IL-8 concentration measured in canine NLR PRBCs in a study by Corsi et al. was ~1300 ± 650 pg/mL after 35 days of storage.\textsuperscript{15} It is unclear why the NLR PRBCs in our study contained more than twice the concentration of IL-8 compared with the results of Corsi et al., but may be attributed to differences in cohorts, measurement methodologies, and blood collection methods. All of our dogs were greyhounds whereas Corsi et al. used random-source research dogs.\textsuperscript{15} We measured IL-8 using a multiplexed genomic and proteomic biomarker analyzer, whereas Corsi et al. used an enzyme-linked immunosorbent assay.\textsuperscript{15} Corsi et al. sedated their dogs with atropine, dexmedetomidine, and butorphanol\textsuperscript{15} compared to sedation with methadone and anesthesia with alfaxalone and isoflurane in our study. Most of our greyhounds also received intermittent positive pressure ventilation during the anesthetic. Some of these differences may have influenced cytokine release. Studies in rodents have found that mechanical stretch of lung parenchyma associated with positive pressure ventilation resulted in activation of inflammatory molecular pathways with inflammatory cell infiltration into lung tissue.\textsuperscript{23,24} Hyperoxia associated with inspiring 100% oxygen is another possible cause of up-regulation of the immune response, and has been found to up-regulate the production of inflammatory cytokines including TNF-α and IL-8.\textsuperscript{25,26} Corsi et al. reported IL-8 concentrations in the NLR units increased significantly compared with the LR units on days 28 and 35\textsuperscript{15} while our study showed IL-8 was synthesized rapidly and early, with significantly increased concentrations in NLR units from day 0 onwards. It is theoretically possible that a genetic tendency in greyhounds exists, causing early activation of leukocytes with increased synthesis of large quantities of IL-8.
compared with other dog breeds. Further research may determine any breed specific differences in cytokine production, or if different sedation and anesthetic protocols during blood collection impact on cytokine production.

Tumor necrosis factor-α and IL-1β did not increase significantly during storage in any of our PRBC units which is consistent with the study by Corsi et al.\textsuperscript{15} In that study, IL-1β and TNF-α concentrations were higher in all NLR units compared with LR units, but these differences did not reach statistical significance. Similarly, the concentration of IL-1β in NLR units in our study was consistently higher than in LR units, and the failure to find a significant difference in these concentrations may be due to our small sample size. In contrast to the study by Corsi et al., our study found TNF-α was below detectable concentrations in all blood units. Studies in people have shown variable TNF-α and IL-1β concentrations in PRBCs.\textsuperscript{9-12,22} One of the major cytokines implicated in febrile non-hemolytic transfusion reactions is IL-1β, a potent pyrogen. The fact that IL-1β did not accumulate in canine PRBCs may explain why a febrile response after transfusion has rarely been reported in dogs. A recent retrospective study of 211 PRBC transfusions in dogs reported an incidence of 24% for febrile non-hemolytic transfusion reactions.\textsuperscript{27} The criterion to diagnose a febrile non-hemolytic transfusion reaction in this study was a rectal temperature of greater than 39°C during or after the PRBC transfusion. This is in contrast with the definition of febrile non-hemolytic transfusion reactions in people, which is defined as an elevation in temperature of at least one degree Celsius associated with transfusion.\textsuperscript{28} By using an absolute temperature instead of a change in temperature, this study may have over-reported the occurrence of febrile reactions as some of the dogs may have had an elevated temperature prior to transfusion commencement. In people, the frequency of febrile non-hemolytic transfusion reactions is reported to occur in 0.2 to 6.8% of red blood cell transfusions and 18 to 37.5% of platelet transfusions.\textsuperscript{29-31} These reactions are a common cause of increased morbidity in people receiving transfusions as they experience significant discomfort with the onset of fever,
chills and rigors. There are a number of issues that make determining the frequency of febrile non-hemolytic transfusion reactions in animals difficult. Not all transfusion recipients have their temperatures closely monitored, causing the frequency of these reactions to be under-reported. It has also been demonstrated that dogs can develop an inflammatory response after transfusion that is not always associated with a febrile response. Similarly in people, a study of 82 transfusion incidents found 28 were associated with chills, rigors and discomfort, consistent with febrile non-hemolytic transfusion reactions, but only 3 of these people were febrile.

Leukoreduction has been performed in dogs in only a small number of studies. Brownlee et al. effectively leukoreduced canine PRBCs and reported no adverse effect on the viability of canine red blood cells that had undergone filtration. The leukoreduction performed in our study was successful at reducing the leukocyte concentration to less than $0.02 \times 10^6$ cells/L. The Food and Drug Administration (FDA) recommends a residual leukocyte count of $<5.0 \times 10^6$ per unit of human PRBCs. Other studies have performed manual leukocyte counts to more accurately determine the residual leukocyte count. Our leukocyte counts were well below FDA recommendations and automated counts were considered adequate.

Leukoreduction prevented accumulation of IL-8 in our study. Other studies have shown leukoreduction reduced other bioactive substances in human transfusion products including myeloperoxidase, eosinophil cationic protein, histamine, and plasminogen activator inhibitor-1. In dogs, leukoreduction was found to prevent the accumulation of inflammatory microparticles in one study, and vascular endothelial growth factor in another. Other benefits of leukoreduction in people include reduction in febrile non-hemolytic transfusion reactions, reduced mortality, decreased incidence of transfusion related acute lung injury, decreased incidence of post-operative infections, and a reduction in the transmission of infectious diseases such as cytomegalovirus.
Leukoreduction increases the cost and preparation time of producing PRBCs. However, in light of preventing accumulation of IL-8 and the potential for removal of other inflammatory mediators not yet evaluated, leukoreduction may be cost effective if it reduces patient morbidity and mortality. Further large scale clinical studies are needed to determine the role of inflammatory mediators on transfusion morbidity in dogs.

There were a few limitations in our study. While a strength of our study was low variability between LR and NLR units, the sole use of greyhounds for our blood units may limit the generalizability of our results. We do not know if greyhound blood products contain more IL-8 than blood products from other dog breeds. Despite this limitation, the results obtained are important to consider. Greyhounds are often used as blood donors because their blood antigen profile more commonly makes them universal donors compared with non-Greyhound dogs and their high PCV makes them red cell-rich donors. The generalizability of our results may also be restricted since the dogs in our study were anesthetised and ventilated, which may have impacted cytokine release. While our study was sufficiently powered to detect differences in IL-8, it was underpowered to detect any differences in IL-β between NLR and LR, or in NLR over time. The changes seen in IL-β are small and would require sample sizes in excess of 40 to detect significant differences. The relevance of these small differences is not known. Finally, we chose to assess for the accumulation of specific cytokines in canine stored PRBCs based on findings in human stored PRBCs. There are likely other potential mediators of inflammation that accumulate over time in canine stored blood products for which we did not test. However, based on the clear difference between LR and NLR units for IL-8, we would hypothesize that leukoreduction would also reduce the accumulation of these mediators.
3.6 Conclusion

In conclusion, our study confirms that the inflammatory mediator IL-8 is present and accumulates in canine PRBCs, and leukoreduction prevents the accumulation of IL-8 in canine PRBCs. The clinical relevance of administration of blood products containing this inflammatory cytokine is unknown.

3.7 Footnotes

a Fenwal Whole Blood CPDA-1 Triple Blood-Pack Unit, IL, USA.

b Terumo® Imugard® III-RC Leukocyte Removal Filter, Japan.

c Terumo® Teruflex® transfer bag 150ml, Japan.

d Codan Take Set Swan-Lock® needle-free multiple dose access device, Santa Ana, CA.

e Advia 120®by Siemens Healthcare Diagnostics, Norwood, MA.

f Milliplex®MAP Kit from EMD Millipore Corporation, Billerica, MA.

g Magpix®xMAP® from Luminex Corporation, Austin, TX.

h RayBio®Canine IL-1 beta ELISA kit by RayBiotech, Inc., Norcross, GA.

i SAS, version 9.4, SAS Institute Inc, Cary, NC.

3.8 References


4. Chapter 4: Interleukin-8, Interleukin-1β and Tumour Necrosis Factor-α in Sequential Units of Canine Packed Red Blood Cells Collected From Retired Racing Greyhounds

4.1 Abstract

Hypotheses We hypothesised that concentrations of interleukin-8 (IL-8), interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) would increase during storage in the third sequential unit (U3) of canine packed red blood cells (PRBC) collected from terminal donors in haemorrhagic shock. We further hypothesised that leukoreduction would prevent cytokine accumulation in U3 and, lastly, that cytokine concentrations in U3 would be higher than in the first units (U1) collected from the same dogs.

Methods U1 and U3 were each collected from 12 anaesthetised healthy greyhounds. Removal of leukocytes from half of each PRBC unit produced one leukoreduced (LR) and one non-leukoreduced (NLR) unit. Canine IL-8, IL-1β and TNF-α were measured on samples collected from the units during storage on days 0, 10, 20, 30, and 37.

Results The IL-8 concentration in U3 NLR units was significantly higher than day 0 at days 10, 20, 30 and 37, and was significantly higher than LR units at all time points. The IL-1β concentration in U3 did not change over time, or between LR and NLR units. TNF-α was not detected in any unit. There were no significant differences in IL-8 or IL-1β concentration between U3 and U1 at any time point, however some NLR U3 units had markedly elevated IL-8 at day 37 (2060-20682) compared to NLR U1 units (3369-5280).

Conclusion NLR U3 units collected from dogs in haemorrhagic shock showed a significant increase in IL-8 concentration during storage. Leukoreduction was effective at preventing the accumulation of IL-8. There was no difference detected between U3 and U1.
4.2 Introduction

Processing and storage of blood products for use in anaemic or haemorrhaging animals is commonplace in referral veterinary practice. Units of blood are typically collected from healthy, client-owned, non-anaesthetised dogs as part of a community blood donation program, or in some countries, purchased from commercial blood banks. Another source of blood products is from healthy dogs surrendered for euthanasia. These animals are anaesthetised and multiple units of blood are collected from each dog prior to euthanasia. This allows veterinary hospitals to stock stored blood for clinical use.

Inflammatory cytokines have been shown to accumulate during storage in blood units collected from human and canine donors, as well as from euvolaemic anaesthetised terminal canine donors. Interleukin-1β, IL-8, and TNF-α are cytokines that increase in human whole blood and packed red blood cells (PRBCs) during storage. Recently, we demonstrated that stored units of canine PRBCs collected from euvolaemic anaesthetised dogs accumulate IL-8 during storage. An additional study in non-anaesthetised dogs had similar findings.

Terminal donors develop haemorrhagic shock during blood donation, which may induce inflammation. A recent study in anaesthetised greyhounds used as terminal blood donors showed significant changes in blood pressure, cardiac index, and oxygen extraction ratio, consistent with shock. Removal of 32 mL/kg, or approximately two blood units, induced moderate shock and removal of 48 mL/kg, or approximately three blood units, induced severe shock. Acute haemorrhage in rats, pigs and people has been shown to cause an increase in inflammatory cytokine concentrations, such as interleukin-6, IL-8, IL-1β and TNF-α, in as little as 30 to 60 minutes after bleeding. It is unknown if similar increases occur in dogs with haemorrhagic shock, or if these cytokines are increased in blood products collected from anaesthetized terminal canine donors.
Leukoreduction prior to storage is effective in removing leukocytes from human and canine PRBCs and preventing cytokine accumulation during storage.\textsuperscript{1,3-6} Both human and canine recipients of non-leukoreduced (NLR) PRBCs have evidence of enhanced inflammation compared to those that receive leukoreduced (LR) PRBCs.\textsuperscript{12,13} The use of LR blood products has also been associated with lower mortality compared with NLR blood products in people.\textsuperscript{14,15} If sequentially collected units of blood from the same donor have increased cytokines due to development of haemorrhagic shock, leukoreduction may be effective in preventing their accumulation.

The aim of this study was to measure IL-8, IL-1β and TNF-α during storage in the third sequentially collected units of both LR and NLR PRBCs. It was hypothesised that these cytokines would accumulate over time in the NLR PRBCs, and that leukoreduction would prevent their accumulation in LR units. In addition, concentrations of IL-8, IL-1β and TNF-α in the third unit (U3) were compared with historically collected data from the first unit (U1). It was hypothesised that their concentration would be greater in U3 than U1.

4.3 Materials and Methods

This work represents part of a previous study and the methodology described below is identical to the previously published study.\textsuperscript{5}

\textit{Animals}

Twelve healthy ex-racing greyhounds that had been donated to the university for euthanasia were used for this study. There were 7 male and 5 female greyhounds aged between 17 months and 3 years. They were determined to be healthy based on physical examination performed by a staff veterinarian. All dogs had a complete blood count within
reference intervals and were blood typed DEA 1.1 negative. The study protocol was approved by the Institutional Animal Ethics Committee (R2564/13).

**Blood collection and processing**

Each greyhound was sedated with intramuscular methadone (0.3mg/kg). A 20 gauge intravenous catheter was placed in the cephalic vein. Anaesthesia was induced with intravenous alfaxalone (2.3 to 3mg/kg), and maintained with isoflurane (0.5 to 2.5 %) in oxygen delivered via an endotracheal tube. Anaesthetic depth, heart rate, respiratory rate, and blood pressure were monitored. Manual positive pressure ventilation was performed during periods of apnoea.

The right lateral cervical region was clipped of hair and aseptically prepared. The carotid artery was catheterised with a 14 gauge IV catheter capped with a sterile injection port, using a surgical cut-down approach. The needle from the blood collection system (Fenwal Whole Blood CPDA-1 Triple Blood-Pack Unit, Fenwal Inc, Lake Zurich, Ill, USA) was inserted into the capped port, and 450 mL of blood was collected into the sterile collection bag, which contained 63 mL of citrate, phosphate, dextrose, and adenine anticoagulant solution. A total of three units (total 1,350 mL) of whole blood were collected from each dog prior to euthanasia. The dogs were euthanised via IV injection of pentobarbital sodium while under anaesthesia. The U3 bags were used in this study. The second units collected were donated to the hospital’s blood bank for clinical use. The U1 bags were part of a prior study and provided historical data for comparison to U3.

The blood units were weighed on a gram scale and were then centrifuged (at relative centrifugal force of 2,000) for 20 minutes at 4°C, and a plasma extractor (Terumo Separation Stand, Teruflex, ACS-201, Terumo Corp, Tokyo, Japan) was used to transfer the
plasma into a satellite bag. The plasma was frozen and donated to the blood bank for clinical use. The seal of the integrally attached bag was then broken and the red blood cell preservative contained within, 100 mL of additive solution 3, was added to the unit of PRBCs. To reduce variability between samples, each PRBC unit was then divided into 2 equal parts; one of these was passed through a leukoreduction filter (Terumo® Imugard® III-RC Leukocyte Removal Filter, Japan) into a separate storage bag (Terumo® Teruflex® transfer bag 150ml, Japan) to produce one LR unit, and the other half was passed unfiltered into a separate storage bag (Terumo® Teruflex® transfer bag 150ml, Japan) to produce one NLR unit (see Figure 1). The leukoreduction filter and separate storage bags were not integrally attached, rather they were inserted into the ports of the PRBC bag in an aseptic manner. Each LR and NLR unit was weighed with a gram scale; the original bag weight was then subtracted to calculate the volume of blood lost to the leukoreduction filter. All 48 units (12 U1 LR, 12 U3 LR, 12 U1 NLR and 12 U3 NLR) were stored for 37 days at 2 to 6°C and were mixed by inversion every 24 to 48 hours.
**Sampling of prepared PRBC units**

A blood bag sampling spike (Codan Take Set Swan-Lock® needle-free multiple dose access device, Santa Ana, CA) was aseptically inserted into each LR and NLR PRBC bag (n=48) port for repeated sample collection during the study. A 12 mL blood sample was aseptically collected from each bag immediately prior to storage (day 0); 2 mL was run through an automated haematology analyzer (Advia 120® by Siemens Healthcare Diagnostics, Norwood, MA) to obtain a CBC; the remaining 10 mL sample was centrifuged at 3,000 RPM for 15 minutes, and the supernatant stored at -80°C. Additional 10 mL samples were aseptically collected from each bag on days 10, 20, 30, and 37 (Figure 1). All of these samples were centrifuged as described, and supernatant were stored at -80°C for later cytokine analysis.

Sample collection was performed over a 2 month period. After the final sample was collected from each bag, the units were kept stored at 2° to 6°C, and once all samples had been collected, an additional 4 mL sample was aseptically collected from each bag. Each final sample was transferred to a culture medium containing 20 mL of peptone-enriched tryptic soy broth supplemented with brain-heart infusion solids and activated charcoal (BacT/ALERT PF Pediatric FAN, bioMerieux, Marcy l’Etoile, France) and sent to a microbiology laboratory (Vetpath laboratory Services, Ascot, WA, Australia) for aerobic and anaerobic bacterial culture.

**Cytokine assessment**

Concentrations of TNF-α and IL-8 were assessed using a canine cytokine magnetic bead kit (Milliplex® MAP Kit from EMD Millipore Corporation, Billerica, MA) in the authors’ in-house multiplexed genomic and proteomic biomarker analyzer (Magpix®xMAP® from Luminex Corporation, Austin, TX). Canine IL-1β was measured using a commercial ELISA kit (RayBio®Canine IL-1 beta ELISA kit by RayBiotech, Inc., Norcross, GA). These tests were
performed according to the manufacturers’ guidelines. A calibration and verification procedure was performed prior to sample analysis. Quality controls and serial dilution standards were prepared according to the manufacturers’ guidelines. The quality controls were within the appropriate range for each assay.

**Statistical analysis**

Statistical analysis was performed with commercially available software (SAS, version 9.4, SAS Institute Inc, Cary, NC). The bag weights, platelet counts and haematocrit of each unit were normally distributed, verified by failure to reject the null hypothesis of normality (Shapiro-Wilk test, P>0.05) and were analysed using a paired t-test (P<0.05). The concentrations of IL-1β, and IL-8 were positively skewed and failed to follow a normal distribution, verified by rejection of the null hypothesis of normality using the Shapiro-Wilk test (P < 0.05). The concentrations are presented as medians (interquartile range). An inverse transformation of the IL-1β and IL-8 concentration created a normally distributed response, verified by failure to reject the null hypothesis of normality using the Shapiro-Wilk test (P > 0.05). The inverse concentration of IL-1β and IL-8 were each analysed using a mixed effect model, evaluating the fixed effects of blood units (LR, NLR) and time (day 0, 10, 20, 37) accounting for the random variance of dogs across paired blood units and the repeated time points. Where there was significant interaction of blood units and time (at P ≤ 0.05), selected posthoc comparisons were made across blood units and time points using a Scheffe-adjusted P ≤0.05.

Comparison of results from this study were made to previous data collected from U1⁵. Characteristics of the units (bag weight, haematocrit, leukocyte counts, and platelet counts) were compared across unit numbers (U3 vs U1) using paired t-tests with significance determined at P<0.05. The inverse concentration of IL-1β and IL-8 were compared using a mixed model including the fixed effects of blood units (LR, NLR), unit (3
vs 1) and time (day 0, 10, 20, 37) accounting for the random variance of dogs across paired blood units and the repeated time points. Where there was significant interaction of blood units, unit number and time ($P \leq 0.05$), selected posthoc comparisons were made across blood units, unit number and time points using a Scheffe-adjusted $P \leq 0.05$.

### 4.4 Results

There was no difference in bag weight or haematocrit between NLR and LR units (Table 1). The mean (SD) weight of blood lost to the leukoreduction filter was 46g (7g). Leukocyte counts ranged between $1.7 \times 10^9$ cells/L and $8.4 \times 10^9$ cells/L in NLR units and were below the detectable limit in all units post leukoreduction. This translates to a leukocyte count of $<0.00002 \times 10^9$ cells/L, which equates to a leukocyte reduction of $>99.9\%$ in all LR units.

Platelet counts were significantly reduced in LR units when compared with NLR units (Table 1). Bacterial cultures showed no growth for all units.

There was a significant interaction of blood units and time for IL-8 ($P=0.0001$). Posthoc comparisons showed no significant differences for IL-8 concentration in U3 LR units between day 0 and later time points. The IL-8 concentration in U3 NLR units was significantly higher than day 0 at time points 10 ($P = 0.04$), 20 ($P = <0.001$), 30 ($P = <0.001$)

### Table 1: Bag characteristics of non-leukocyte-reduced and leukocyte-reduced units of packed red blood cells collected from terminal greyhounds after the onset of haemorrhagic shock (Unit 3).

<table>
<thead>
<tr>
<th></th>
<th>NLR Units</th>
<th>LR units</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td>173g (14g)</td>
<td>177g (10g)</td>
<td>0.58</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.69 (0.03)</td>
<td>0.67 (0.03)</td>
<td>0.54</td>
</tr>
<tr>
<td>Platelet Count ($10^9$/L)</td>
<td>219.67 (49.91)</td>
<td>4 (1.60)</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>
and 37 (P < 0.001) (Table 2). There was no significant interaction of blood units and time for IL-1β (P=0.116), so post hoc comparisons were not made. Thus, the IL-1β concentrations were not significantly different between U3 LR and NLR units, and did not change significantly over time (Table 3). The TNF-α concentrations were less than the minimum detectable value (6.1 pg/mL) in all U3 NLR and LR units at all time points so analysis was not performed.

**Table 2:** Median (interquartile range) interleukin-8 concentrations (pg/mL) in leukoreduced (LR) and non-leukoreduced (NLR) units of packed red blood cell units at time points throughout storage. Units were sequentially collected from individual dogs, however, only the first (Unit 1) and third (Unit 3) units from each donor were analysed.

<table>
<thead>
<tr>
<th>Unit 3</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Day 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 LR*</td>
<td>320 (128-1554)</td>
<td>327 (104-3168)</td>
<td>337 (134-3228)</td>
<td>427 (96-3740)</td>
<td>402 (123-3922)</td>
</tr>
<tr>
<td>IL-8 NLR†</td>
<td>1675 (541-6210)A</td>
<td>3608 (1500-27401)B</td>
<td>8187 (1787-25616)B,C</td>
<td>11935 (1223-33148)C</td>
<td>6025 (2060-20682)B,C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unit 1+++</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Day 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 LR*</td>
<td>316 (222-425)</td>
<td>353 (267-436)</td>
<td>436 (338-512)</td>
<td>452 (390-568)</td>
<td>472 (361-597)</td>
</tr>
<tr>
<td>IL-8 NLR†</td>
<td>1606 (1149-2314)A</td>
<td>4005 (2334-5693)B</td>
<td>4838 (2778-6060)B</td>
<td>5367 (3003-7167)B</td>
<td>4346 (3369-5280)B</td>
</tr>
</tbody>
</table>

* There were no differences for IL-8 LR over time.

† IL-8 concentrations in NLR significantly higher than in LR on all days (P < 0.001)

A,B,C Within a row, days with the same superscript are not significantly different (Scheffe adjusted P > 0.05)

Table 3: Median (interquartile range) interleukin-1β concentrations (pg/mL) in leukoreduced (LR) and non-leukoreduced (NLR) units of packed red blood cell units at time points throughout storage. Units were sequentially collected from individual dogs, however, only the first (Unit 1) and third (Unit 3) units from each donor were analysed.

<table>
<thead>
<tr>
<th>Unit 3</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Day 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β LR*</td>
<td>29 (15-204)</td>
<td>28 (18-185)</td>
<td>29 (19-184)</td>
<td>25 (17-169)</td>
<td>26 (17-145)</td>
</tr>
<tr>
<td>IL-1β NLR*</td>
<td>32 (17-184)</td>
<td>33 (20-222)</td>
<td>41 (19-180)</td>
<td>33 (20-215)</td>
<td>36 (21-180)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unit 1†</th>
<th>Day 0</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
<th>Day 37</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β LR*</td>
<td>17 (0-25)</td>
<td>17 (2-30)</td>
<td>15 (4-30)</td>
<td>17 (3-37)</td>
<td>13 (5-35)</td>
</tr>
<tr>
<td>IL-1β NLR*</td>
<td>48 (13-62)</td>
<td>43 (23-87)</td>
<td>36 (24-78)</td>
<td>48 (19-107)</td>
<td>71 (24-92)</td>
</tr>
</tbody>
</table>

* There were no differences for IL-1β over time or between LR and NLR over time.


Statistical analysis of data with that from the original study allowed comparison between U3 and U1 (Table 4). The mean (SD) leukocyte counts in U3 NLR units were significantly lower than in U1 NLR units. There were no significant interactions of unit and time for IL-8 (P=0.666) or IL-1β (P=0.367) concentrations so post hoc comparisons were not made. Thus, concentrations were not significantly different in U1 NLR units compared with U3 NLR units at any time points.
Table 4: Comparison of bag characteristics of non-leukocyte-reduced and leukocyte-reduced units of packed red blood cell units collected from terminal greyhounds prior to the onset of haemorrhagic shock (Unit 1) and after induction of haemorrhagic shock (Unit 3).

<table>
<thead>
<tr>
<th></th>
<th>Unit 1</th>
<th>Unit 3</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean NLR bag weight (SD)</td>
<td>173g (13g)</td>
<td>173g (14g)</td>
<td>0.25</td>
</tr>
<tr>
<td>Mean LR bag weight (SD)</td>
<td>180g (6g)</td>
<td>177g (10g)</td>
<td>0.26</td>
</tr>
<tr>
<td>Volume lost to the LR filter</td>
<td>47g (5g)</td>
<td>46g (7g)</td>
<td>0.71</td>
</tr>
<tr>
<td>Haematocrit in NLR units</td>
<td>0.68 (0.03)</td>
<td>0.69 (0.03)</td>
<td>0.90</td>
</tr>
<tr>
<td>Haematocrit in LR units</td>
<td>0.67 (0.03)</td>
<td>0.67 (0.03)</td>
<td>0.86</td>
</tr>
<tr>
<td>Platelet count in NLR units (10^9/L)</td>
<td>215.8 (44.58)</td>
<td>219.67 (49.91)</td>
<td>0.84</td>
</tr>
<tr>
<td>Platelet count in LR units (10^9/L)</td>
<td>3.5 (2.02)</td>
<td>4 (1.60)</td>
<td>0.51</td>
</tr>
<tr>
<td>Leukocyte count in NLR units</td>
<td>6.23 (2.45)</td>
<td>4.67 (2.25)</td>
<td>0.009</td>
</tr>
<tr>
<td>Leukocyte count in LR units</td>
<td>0</td>
<td>0</td>
<td>0.16</td>
</tr>
</tbody>
</table>

4.5 Discussion

Our results confirmed our first hypothesis that units of PRBCs collected late in the bleeding process, when donors are in shock, accumulate IL-8 during storage. Similar to our previous study, the concentration of IL-8 in NLR U3 significantly increased over time, whereas there was no elevation in IL-1β over time and TNF-α was undetectable at all time points. Our results also confirmed our second hypothesis, that leukoreduction would prevent accumulation of inflammatory cytokines over time. We found that leukoreduction significantly reduced IL-8 concentrations, but found no difference in IL-1β concentrations between NLR and LR U3 at any time point. Our results did not confirm our third hypothesis.
that cytokine concentrations in U3 would be significantly higher than in the first units (U1) collected from the same dogs. Analysis of NLR U3 in this study showed no concentration difference in any of these cytokines, compared to NLR U1 in our first study.\(^5\)

In our previous study, we found IL-8 concentration to increase over time in NLR U1 PRBCs.\(^5\) In this study we hypothesised that there would be further increases in IL-8 as well as increased IL-1β and TNF-α concentrations in U3, due to the development of haemorrhagic shock. An experimental study in rats subjected to trauma and haemorrhage found that TNF-α levels were significantly elevated 45 minutes into haemorrhage.\(^8\) The authors felt that the elevation in TNF-α was mostly due to haemorrhage but could not rule out an effect of trauma. Another study in mice by the same authors found that haemorrhage alone, without trauma, resulted in significantly elevated levels of TNF-α at 30 minutes into hemorrhage.\(^9\) A study in humans found that patients with haemorrhagic shock secondary to ruptured abdominal aortic aneurysm had significantly elevated TNF-α and IL-1β at hospital admission.\(^11\) This increase was significantly greater than in trauma patients. A recent study in anaesthetised pigs with induced liver injury and haemorrhage found that levels of interleukin-6, IL-8 and TNF-α were all significantly elevated from baseline to 1 hour and from baseline to 2 hours.\(^10\) These studies suggest that there is a rapid increase in inflammatory mediators following haemorrhage. We therefore found it surprising that the IL-8, IL-1β and TNF-α concentrations in U3 were not significantly elevated when compared to U1.

One reason for no differences between U3 and U1 may have been due to the degradation of cytokines between blood collection, processing, and storage. Tumour necrosis factor-alpha, for example, has a very short half-life of 6 to 20 minutes in humans and laboratory animals.\(^17\) Sample processing needs to occur rapidly after collection in order to preserve TNF-α. As multiple units were collected from multiple dogs on the same day, and all units
were processed at the end of blood collection, the time delay between collection and processing of the blood units may have resulted in a reduction of TNF-α in PRBC units in our study. If this is the case however, TNF-α accumulation would not be a concern for our transfusion recipients as it is unlikely to accumulate in transfusion products over time. A second possibility for the lack of difference in TNF-α may be that our assay was not sensitive enough to show elevations in TNF-α. The detection limit for TNF-α for our in-house multiplexed genomic and proteomic biomarker analyser was 6.1 pg/mL. Many studies evaluating human TNF-α in human blood products use assays that are much more sensitive with a detection limit of 0.17 pg/mL to 0.7 pg/mL. Another possible reason for the lack of a significant increase in cytokine concentration between U1 and U3 may have been due to the short time spent in haemorrhagic shock. Blood collection in most of these dogs was performed within 20 minutes after induction of anaesthesia, and collection of U3 was complete within 40 minutes after induction of anaesthesia. Although these dogs were showing signs of haemorrhagic shock, such as weak femoral pulse quality, pale mucous membrane colour and tachycardia, there may not have been enough time to induce a marked inflammatory response.

Although the concentration of IL-8 was not significantly higher in U3 than in U1, the results showed marked variation in IL-8 concentration between all NLR units. Therefore, although not significantly different due to a large overlap between groups, there is a marked variance in IL-8 concentration between individual NLR units. In selecting a NLR U3 it is possible to transfuse a unit with IL-8 in excess of 20,000 pg/mL. The administration of a PRBC unit with a very high concentration of IL-8 may contribute to a patient’s systemic inflammation when compared with a unit containing less IL-8. Why there was such a large variance in IL-8 concentration between individual NLR units is not known, however our study did consistently show that leukoreduction was able to significantly reduce the concentration of inflammatory cytokines in canine PRBCs. The LR units in our study had
significantly less IL-8, and there was less variation between units. Therefore, selection of an
LR unit at any time throughout storage would consistently result in the transfusion of fewer
cytokines.

The leukocyte counts in U3 NLR units were significantly less than the leukocyte counts in
U1 NLR units. The reduction in leukocytes in U3 NLR units may be due to cytokine release
during haemorrhagic shock enhancing neutrophil-endothelial interactions. An
experimental study in dogs that underwent acute haemorrhagic shock showed a drop in
peripheral leukocyte count to 20% of control values. The control dogs underwent
anaesthesia without being bled. They found that neutrophils had accumulated within the
pulmonary vessels, with a 60% increase in neutrophils within the pulmonary blood vessels
when compared with controls. A study in rats with a loss of 30% of blood volume showed
significant accumulation of leukocytes within the pulmonary circulation associated with the
formation of microthrombi. It is possible in our study that haemorrhagic shock caused
pulmonary leukocyte accumulation, leading to reduced leukocyte counts in U3.

Our study showed that leukoreduction was able to prevent accumulation of IL-8 during
storage of sequentially collected PRBC units. The leukoreduction performed in our study
was successful at reducing the leukocyte concentration to less than 0.02 x 10^6 cells/L. The
Food and Drug Administration (FDA) recommends a residual leukocyte count of <5.0 x 10^6
per unit. Other studies have performed manual leukocyte counts to more accurately
determine the residual leukocyte count. Our leukocyte counts were well below FDA
recommendations and automated counts were considered adequate. Leukoreduction does
add to the cost of blood processing, with each filter costing approximately $25 as well as an
increase in processing time. Many studies have verified the ability of leukoreduction to
reduce bioactive substances in human and canine transfusion products. The benefits of
leukoreduction in people include a reduction in febrile non-haemolytic transfusion
reactions, decreased incidence of transfusion related acute lung injury, decreased incidence of post-operative infections, a reduction in the transmission of infectious diseases, and reduced mortality. The benefit of administering LR PRBC units in dogs has not been demonstrated, however preventing accumulation of inflammatory mediators such as IL-8 during storage may be beneficial if it reduces recipient morbidity and mortality. This may be particularly applicable to sequentially-collected units from terminal donors, where we saw extremely high IL-8 concentrations in some units. Further large-scale clinical studies are needed to determine the role of storage-related inflammatory mediators on transfusion morbidity in dogs.

There were a few limitations in our study. The sole use of greyhounds for our blood units may limit the generalisability of our results. We do not know if greyhound blood products contain more IL-8 than blood products from other dog breeds. Despite this, we feel the evaluation of greyhound blood is clinically relevant, as they are often used as blood donors because their blood antigen profile more commonly makes them universal donors compared with non-greyhound dogs. They are also likely the only breed, to the authors’ knowledge, used in terminal blood donor programs. The dogs in this study were not instrumented for cardiac output monitoring, therefore the level of shock was presumptive. However, these dogs were bled using the same protocol as a previous study by the same group and we are confident of the degree of shock when the third bag was collected. While our study was sufficiently powered to detect differences in IL-8, it was underpowered to detect any differences in IL-1β between NLR and LR, or in NLR over time. The changes seen in IL-1β are small and would require a sample size in excess of 40 to detect a significant difference, however, these small differences may simply reflect normal variability. A further limitation is the time delay that occurred between collection of our blood units, processing and sampling, which may have altered cytokine concentrations. In our study, we collected blood into citrate, phosphate, dextrose, and adenine anticoagulant
solution with the addition of additive solution 3 preservative solution. There are other available anti-coagulant solutions and preservatives used in blood processing, and the effect of other solutions on cytokine production is unknown. Finally, we chose to assess for the accumulation of specific cytokines in canine stored PRBCs based on findings in human stored PRBCs. There are likely other potential mediators of inflammation that accumulate over time in canine stored blood products for which we did not test. However, based on the clear difference between LR and NLR units for IL-8, we hypothesize that leukoreduction would also reduce the accumulation of these mediators.

In conclusion, our study showed a significant increase in IL-8 concentration in U3 NLR units during storage. The IL-8 concentrations in NLR units were also extremely variable and in some units markedly elevated. When the concentrations of cytokines in U3, i.e. dogs in haemorrhagic shock, were compared with cytokine concentrations in U1, no significant differences were detected. Leukoreduction was effective in preventing the accumulation of IL-8 in all units. These findings support the use of leukoreduction to prevent the accumulation of inflammatory mediators in stored canine PRBCs, especially for units from terminal blood donors. Preventing accumulation of these inflammatory mediators may have beneficial effects on patient morbidity and mortality, although the effects of infusion of these mediators at this time are unknown.

Acknowledgement

The authors acknowledge Dorian Lara’s assistance in blood collection and processing.

4.6 References


Relation with subsequent adult respiratory distress syndrome and multiple organ failure. 


5. Chapter 5: Conclusion

Our research has confirmed that inflammatory cytokines accumulate in canine PRBC during storage. We found that IL-8 accumulated in canine PRBC during storage, and we successfully demonstrated that leukoreduction was effective at preventing accumulation of IL-8. These findings are consistent with a recent study in dogs. Our research however was unique in that we investigated cytokine accumulation in PRBC collected from terminal greyhounds in haemorrhagic shock. Ex-racing greyhounds are regularly donated to our hospital and used as terminal blood donors. During terminal donation these dogs develop haemorrhagic shock which may affect the quality of the blood products collected. Studies in people, pigs and rodents have found elevated cytokine concentrations following acute haemorrhage. We hypothesised that PRBC collected from dogs with haemorrhagic shock would have elevated concentrations of cytokines compared with PRBC collected from the same dogs prior to the development of haemorrhagic shock. Our study was unable to demonstrate significantly higher IL-8, IL-1β or TNF-α in PRBC collected from donors in haemorrhagic shock compared with euvoalaemic donors, however the concentration of IL-8 in U3 PRBC units was extremely variable, and some units had markedly elevated IL-8.

Cytokines that accumulate within blood transfusion products contribute to inflammation in human transfusion recipients. Clinically this may become evident as transfusion reactions, such asFNHTR and TRALI. We are currently in the process of designing a clinical study to investigate the clinical effect of transfusing cytokine-rich blood products to critically ill dogs. We hope to start answering the question as to whether or not these cytokines are associated with increased canine morbidity and mortality. A likely problem that we will encounter is the marked variability in cytokines between individual units of PRBC, individual patients before transfusion, and also the effect underlying diseases will have on cytokine concentrations.
By removing inflammatory cytokines from blood products, leukoreduction has been found to reduce the occurrence of adverse effects and even mortality in people.\textsuperscript{135,136,139,143}

Clinical studies in dogs investigating leukoreduction have not been performed. Further studies are warranted to assess the effect of leukoreduction on overall canine transfusion recipient morbidity and mortality. Unfortunately, transfusion reactions that leukoreduction may prevent including FNHTR and TRALI are not commonly reported in dogs, and the number of clinical patients required to find a significant difference with leukoreduction may be a limiting factor.

Our research has confirmed that inflammatory cytokines accumulate in canine PRBCs collected from euvalaemic donors and donors in haemorrhagic shock. Leukoreduction is an effective method of removing white blood cells from canine PRBCs and preventing the accumulation of inflammatory cytokines. Although further clinical studies are require, leukoreduction is likely an effective and cost-effective method of reducing cytokine induced transfusion reactions.
Concentrations of TNF-α and IL-8 were assessed by the author using a canine specific cytokine magnetic bead kit in the authors’ in-house multiplexed genomic and proteomic biomarker analyzer. This technology uses colour-coded magnetic microspheres coated with specific capture antibodies. The samples are added and the analyte is captured by the beads. A biotinylated detection antibody is then introduced and the samples are incubated with Streptavidin-PE conjugate. The multiplex analyser then uses light-emitting diodes and a CCD camera to detect the marker of interest. The technology is thus similar to an enzyme-linked immunosorbent assay (ELISA), however multiple analytes can be measured simultaneously which reduces sample size, processing time and cost. A canine specific cytokine magnetic bead panel was sourced for measurement of TNF-α and IL-8 for use with the multiplex analyser. The limit of detection for the TNF-α assay was 6.1 pg/mL, with an intra-assay coefficient of variation (CV) of <5%. The limit of detection for the IL-8 assay was 21.7 pg/mL, with an intra-assay CV of <5%.

Assays were performed in accordance with the manufacturer’s instructions. A calibration and verification procedure was performed prior to sample analysis. Plasma samples were thawed to room temperature and were mixed by vortexing and then centrifuging to remove particulate matter. The antibody-immobilised beads were prepared firstly by sonicating for 30 seconds and then vortexing for one minute before adding assay buffer. The quality controls provided in the kit were reconstituted with deionised water and then inverted several times before vortexing to mix. Serum matrix was prepared with the appropriate concentrations of CPDA, SAGM and serum matrix provided. Canine cytokine panel standards were then prepared at the following concentrations; 50000, 12500, 3125, 781, 195, 48.8 and 12.2 pg/mL. Two hundred μL of assay buffer was added to each well of the 96 well plate. The plate was sealed and mixed on a plate shaker for 10 minutes at room temperature.
temperature. The assay buffer was then decanted and the plate was inverted and tapped onto absorbent towels. Twenty five μL of each standard and control were added to the appropriate wells, and 25μL of assay buffer for the standard 0. Twenty five μL of assay buffer was added to each sample well and 25μL of matrix solution was added to the background, standards and control wells. Twenty five μL of each plasma sample was added to each sample well in duplicate. Twenty five μL of the magnetic beads solution was then added to each well. The plate was sealed and wrapped in aluminium foil and incubated overnight at 4°C on a plate shaker. The following day the plate was washed with an automatic plate washer, with the wash buffer provided, for two wash cycles. Twenty five μL of detection antibodies were added to each well, the plate was sealed and covered with aluminium foil and was incubated on a plate shaker for 1 hour at room temperature. Twenty five μL of Streptavidin-Phycoerythrin was then added to each well before covering and incubating again on a plate shaker for 30 minutes at room temperature. The plate was then washed again as previously described. One hundred fifty μL of drive fluid was then added to all wells and incubated for 5 minutes on a plate shaker at room temperature. The plate was then inserted into the multiplex analyser for analysis.

Canine specific IL-1β was measured using a commercial ELISA kit as this analyte was not available using the multiplex technology. The assay was performed pursuant to the manufacturer’s guidelines. The assay uses a 96 well plate coated with canine specific IL-1β antibodies. Bound IL-1β is detected using biotinylated anti-canine IL-1β antibody. The limit of detection of the assay was 10 pg/mL, with an intra-assay CV of <10%.

All plasma samples were thawed at room temperature and vortexed. Standards were prepared at the following dilutions; 8000, 2667, 888.9 296.3, 98.77, 32.92, and 10.97 pg/mL. One hundred μL of each standard and sample were added in duplicate to the appropriate wells. The plate was covered and incubated on a plate shaker at room
temperature for 2.5 hours. The plate was washed with an automatic washer with the provided wash buffer for 4 cycles. The plates were then inverted onto absorbent towel to remove any remaining wash buffer. One hundred μL of biotinylated antibody was then added to each well and the plate was then incubated for a further hour on a plate shaker at room temperature. The solution was then discarded and the wash process repeated as previously described. One hundred μL of Streptavidin solution was then added to each well and the plate was incubated on a plate shaker at room temperature for 45 minutes. The solution was discarded and the wash process again repeated as outlined above. One hundred μL of tetramethylbenzidine one-step substrate reagent was then added to each well and the plate was incubated on a plate shaker at room temperature for 30 minutes. Fifty μL of stop solution was added to each well and the optical density was read at 450nm using an ELISA plate reader.