

Influence of transfusion technique on survival of autologous red blood cells in the dog

Ruth I McDevitt DVM; Craig G Ruaux, BVSc, PhD, DACVIM and Wendy I Baltzer, DVM, PhD
DACVS

From the Department of Clinical Sciences, College of Veterinary Medicine Oregon State
University, Corvallis, OR 97330

Address correspondence and reprint requests to:

Dr. Craig G Ruaux
Dept of Clinical Sciences, College of Veterinary Medicine
Oregon State University
Corvallis, OR 97330
Email:craig.ruaux@oregonstate.edu

Dr. McDevitt's current address is: Ocean States Veterinary Specialists, 1480 South County
Trail, East Greenwich, RI 02818

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Running Title: Transfusion technique and RBC Survival

Abstract

Objective – To determine the effect of 3 differing transfusion techniques on survival of autologous canine red blood cells (RBCs).

Design – Prospective, blinded study.

Setting – University Teaching Hospital.

Animals – Nine healthy dogs.

Interventions – Three distinct preparations of RBCs, each representing ~1% of red cell mass, were generated for each dog by biotinylation of RBCs at varying biotin densities.

Labeled cells were transfused using 3 techniques (gravity, volumetric pump, syringe pump). Serial determinations of red cell survival were carried out by flow-cytometric analysis of RBCs collected at 7-day intervals for 49 days. In vitro analysis of the effect of transfusion methods on RBC integrity and osmotic fragility were carried out in 7/9 dogs.

Measurements and Main Results – RBCs administered via volumetric and syringe pumps exhibited a marked decrease in short-term probability of survival compared to RBCs delivered by gravity flow. At 24 hours, only 4/8 and 1/7 dogs had surviving cell populations delivered by volumetric and syringe pump respectively, compared with 8/8 dogs which had surviving cell populations delivered by gravity flow. Circulating half-life of cells surviving at 24 hours after delivery by volumetric pump was not significantly different to that delivered by gravity flow. No significant effect on in vitro RBC integrity or osmotic fragility was detected in relation to transfusion technique.

Conclusions – Delivery of autologous canine RBCs via mechanical delivery systems was associated with a high risk for early loss of transfused cells.

Keywords: Blood administration, hemolysis, red cell survival, mechanical perfusion

Introduction

Red blood cell transfusions are commonly indicated in veterinary medicine. Whole blood and packed red cell transfusions are indicated in a variety of conditions including tissue hypoxia from blood loss, immune mediated hemolytic anemia, and decreased erythrocyte production due to bone marrow disease. Canine blood for transfusion is generally obtained from in-house or community blood donors, or may be purchased from commercial blood banks. Because of the necessary equipment, trained personnel and specialized handling requirements for safe transfusions, the costs associated with this treatment can be considerable. Thus, it is important to ensure that any red cells collected remain viable prior to and after transfusion, and to optimize delivery techniques to minimize complications and morbidity.

Previous studies have investigated techniques for the collection and storage of canine blood,¹⁻⁴ but to date there has not been a comprehensive study determining the optimal method of administration of red cells to dogs. Ideally, transfusion of red cells should enable personnel to administer blood at a specified rate and volume without causing damage to the cells. Control of administration rate and accurate determination of delivered volume is easier when mechanical pump systems are used, however mechanical damage above and beyond that caused by collection and storage may ensue. Damage to red cells is expected to result in decreased lifespan of the cells, and hence diminished effectiveness of the transfusion.

Three methods of blood administration are commonly used in veterinary medicine. These include administration of blood via gravity flow using a blood administration set with built-in filter, administration using a volumetric peristaltic infusion pump and blood administration set with built-in filter, and administration of blood using a standard syringe

pump with a filter to remove cellular aggregates. Although there is substantial anecdotal evidence and opinion regarding each of these techniques, to date there are no controlled published studies to validate or compare these techniques in dogs.

A number of studies are available in the human literature that compare peristaltic pumps to centrifugal pumps or other methods of administration.⁵⁻⁹ Most of these studies assess damage to red cells *in vitro*, and rarely examine the effect that transfusion administration has on *in vivo* red cell survival. Little consensus exists regarding whether or not damage occurs when a particular method is used, and whether or not this is clinically relevant. Several published studies failed to demonstrate differences in hemoglobin concentration or osmotic fragility when comparing rotary and centrifugal pumps.^{7,8,10-12} By contrast, linear peristaltic pumps have been found to increase the free hemoglobin concentration in other studies.¹¹ Linneweber et al,⁵ reported an increase in the quantity of red blood cell fragments when a roller pump is used in comparison to a centrifugal pump. As many of these studies only assessed *in vitro* parameters, or only involved humans undergoing cardiopulmonary bypass, the clinical significance in dogs is unknown.

The primary objective of the present study was to determine if the method of transfusion has an effect on the lifespan of autologous canine erythrocytes. Additional *in vitro* studies were also carried out to assess the impact of transfusion technique on canine RBC osmotic fragility, RBC count and hemoglobin concentrations in plasma.

Materials and Methods

Subjects

Nine privately owned dogs, including 6 males and 3 females were enrolled into the study with owner consent. Breeds represented included 3 Labrador retrievers, 1 German shorthair pointer, 1 Australian shepherd, and 4 mixed breed dogs, ranging in age from 1.5-9 years of age (median 3 years). Approval for all procedures was obtained from our Institutional Animal Care and Use Committee. All animals were housed and cared for by their owners. Body weights ranged from 18.2-31.5 kg, all dogs were in good health, and up to date on vaccinations and flea and heartworm preventive medications.

Biotinylation of RBCs

Blood (2 mL/kg) was collected from the jugular vein of each dog using standard aseptic venipuncture technique into 150 mL capacity blood collection bags containing 7 mL CPDA anticoagulant/50mL collected.^a The total volume of blood was separated into 3 aliquots, (0.7 mL/kg/aliquot); each aliquot containing approximately 1% of the dog's red cell mass. The blood was centrifuged in sterile 50 mL tubes at 2000 x g relative centrifugal force (RCF) for 10 minutes, the plasma layer was aspirated and saved under refrigeration in sterile 50 mL polyethylene tubes. Saved plasma was eventually used to reconstitute red blood cells (RBCs) after biotin labeling. The RBCs were washed 3 times with a phosphate-buffered saline (PBS, pH 7.4) wash buffer containing 11.1 mmol glucose (buffer osmolarity was 356 mOsm.L). Washed RBCs were then suspended in sufficient wash buffer to yield a 25% suspension of RBCs.

Cells in each aliquot were labeled using biotin-X-NHS,^b prepared as a stock solution at a concentration of 2 mg/mL in PBS following initial suspension in dimethylsulfoxide. The biotinylation buffer was adjusted to pH 5.0 with concentrated HCl just prior to dissolution,

to avoid hydrolysis of biotin.¹³ Each of the 3 aliquots of blood from each dog were labeled using either 30, 75, or 150 µg biotin/mL RBCs by addition of varying volumes of the stock biotin solution, following preliminary experiments (data not shown) that demonstrated that these labeling densities generated 3 distinct visible preparations of cells per dog. The RBCs were incubated with the biotinylation reagent with continuous gentle agitation, the reaction was terminated after 30 minutes by addition of a five-fold volume of wash buffer. The biotinylated RBC's were then washed 4 times in wash buffer and suspended in autologous plasma before being transferred back to 150 mL blood storage bags^c or a 60-mL syringe for transfusion. Bags were labeled "pump" or "gravity" to blind the investigators carrying out transfusion and blood sampling to the biotin concentrations present on each preparation. The packed cell volumes of the final products were not measured, however reconstitution in autologous plasma would be expected to yield a final product similar in packed cell volume to whole blood.

Randomization of transfusion techniques

The transfusion technique to be used for each cell preparation in each dog was randomly allocated by 1 investigator, and the investigators carrying out transfusions and subsequent flow cytometry analysis were blinded to the transfusion technique used for each preparation in each dog until data collection was complete.

Transfusion techniques

Each dog was autologously transfused in sequence, using 3 different transfusion techniques via a single 20-Ga cephalic catheter. The first preparation of biotinylated RBCs

was transfused using a volumetric peristaltic infusion pump^d and standard transfusion line with built-in 170-260 micron filter. The second preparation was transfused using a syringe infusion pump^e with cells delivered through an 18 micron aggregate filter^f, and the final preparation was transfused last via gravity flow using a transfusion line with built-in 170-260 micron filter. Both the infusion pump and syringe pumps were used according to the manufacturer's specifications. Transfusion rates for each method were set at 2 mL/kg/hr, which is the highest transfusion delivery rate used under the standard operating procedure for transfusions used at this institution. Delivery rate of the control preparation was adjusted by regulating drops/minute manually.

Blood sampling and detection of biotin-labeled RBCs

Transfused cells were allowed to equilibrate overnight. The morning following transfusion (day 1), and every 7 days until day 49, a 1.5mL sample of whole blood was obtained from each dog by jugular venipuncture and preserved in EDTA. From each sample, 50 μ L of whole blood was transferred to a microcentrifuge tube and red cells were washed twice using the previously described PBS-based wash buffer. The supernatant was removed and the RBCs suspended with 100 μ L of PBS wash buffer, 2.0 μ L of fluorescein-labeled streptavidin^g (FITC-AV, 1mg/mL stock solution) was added and the cells were agitated for 30 minutes at 37 °C in a bench-top agitator.^h The final working dilution of FITC-AV was 1:50. Following incubation the cells were removed from the agitator and the reaction terminated by addition of 1000 μ L of phosphate buffer wash, the cells were then washed twice in the PBS-based wash buffer. The supernatant was removed, 1000 μ L of

phosphate buffer was added, and the cells were transferred to 5 mL tubes to which an additional 1000 μ L of PBS was added before flow cytometry.

Flow cytometry

Biotin-labeled cells were analyzed using flow cytometry.ⁱ Five hundred thousand cells were evaluated per sample and number vs fluorescence plotted on a log₁₀ scale. Three gates were assigned to quantify the 3 separate population peaks, to allow quantification of each population over time. These gates were applied consistently to all populations throughout sampling time (Figure 1).

Additional *in vitro* experiments:

In order to determine if detectable damage to red cells is occurring during transfusion, additional *in vitro* studies were carried out using 7 of the 9 dogs previously used in the transfusion study, 2 dogs being unavailable due to owner commitments.

Twenty milliliters of blood was collected from the jugular vein using aseptic technique into a syringe containing 2.5mLs of CPDA to prevent coagulation. The blood from each dog was then divided into 3 aliquots and was subjected to the experimental conditions outlined above (syringe pump and filter, peristaltic pump, or gravity flow). Blood was sampled during the initial pass through each delivery technique and submitted for red blood cell and plasma hemoglobin quantification, while another aliquot was used for osmotic fragility testing.

RBC quantification and plasma hemoglobin determination

Red blood cell counts and total hemoglobin were determined by the institution's in-house diagnostic laboratory, using an automated hematology analyzer.^j

Osmotic fragility testing

Osmotic fragility testing was performed using a saline dilution technique.¹⁴ Briefly, a phosphate buffered 10% NaCl stock solution was made, the stock solution was then diluted with distilled water to make a 1% salt solution. Sixteen test tubes were prepared by serial dilution for each treatment for each dog, containing 0.85 to 0.0% NaCl at 0.05% increments. 20 μ L of whole blood was added to each tube and mixed by inversion, then allowed to stand at room temperature for 30 minutes. All tubes were centrifuged at 2,000 x g for 10 minutes. The optical density of the supernatant was read at 540 nm using a spectrophotometer.^k One hundred percent hemolysis was assumed at 0.0% NaCl. The percentage hemolysis of cells exposed to each saline concentration was calculated from the ratio of A_{540} of the supernatant and the absorbance of the 0.0% NaCl supernatant for each dilution series.

Statistical Analyses

Data were analyzed using a combination of an open source statistical programming environment and commercial statistical software.^{l,m}

Red cell population data were analyzed using a general linear model to model expected variables (inter-dog variation, time and transfusion method), followed by 2-way analysis of variance with time and transfusion method as explanatory variables. Osmotic fragility data were analyzed by 2-way analysis of variance with saline concentration and

transfusion method as explanatory variables. Data for RBC counts and hemoglobin were analyzed by one way analysis of variance with transfusion method as the explanatory variable. Post-hoc analyses were carried out using Tukey's multiple comparison test.

Probability of transfusate survival to day 1 was analyzed via contingency table analysis, comparing each of the mechanical techniques (pump and syringe) to the gravity-delivered control preparations. Contingency table analysis was carried out using Fischer's Exact Test, due to expected counts <5 in some cells.

Quantitative recovery of labeled RBC's was analyzed between gravity and pump-delivered groups using the Mann-Whitney test, as data from the pump-delivered group could not be demonstrated to have a normal distribution ($n=4$, insufficient group size).

For all analyses, a P value of <0.05 was considered significant.

Results

Complications of transfusion and sampling

Sampling was discontinued from 1 dog after day 1 as there were no detectable labeled cells from any preparation present. This dog's blood was the last to be processed for biotinylation, and preparation of new reagents was necessary for this specific dog. As the possibility of laboratory error in preparation of reagents could not be ruled out, this dog was removed from all statistical analyses.

Two dogs failed to receive red cells transfused via syringe pump due to coagulation of the blood within the syringe prior to administration; 1 of these dogs being the same dog subsequently removed from analysis due to lack of signal (above). Coagulation was attributed to mixing of refrigerated plasma with room temperature red cells, however this

hypothesis has not subsequently been tested. One dog received incomplete transfusion of all preparations of labeled RBC's due to catheter failure, however this dog did yield detectable populations (at reduced number) on flow cytometry analysis and thus sampling was continued.

Effect of transfusion technique on 24 hour survival of RBCs

Contingency table analysis demonstrated a highly significant difference in the proportion of red cells with the pump and syringe treatments that were detectable the following morning. (Fischer's Exact Test, $P < 0.001$, Table 1). Both syringe and volumetric pump techniques were associated with a loss of labeled RBC population overnight following transfusion. Only 4/8 dogs displayed a viable population at day 1 that had been delivered via perfusion pump. Only 1 of 7 populations delivered via syringe pump was detectable at day 1. All cell preparations (8/8) delivered by gravity flow yielded detectable viable cell populations after transfusion.

Effect of transfusion technique on quantitative RBC recovery

There was no significant effect ($P = 0.904$, Mann-Whitney Test) of transfusion technique on quantitative recovery of labeled RBC's in the gravity and volumetric pump groups at day 1. Median counts of labeled cells by delivery method were 4,289 (Gravity, $n = 6$) and 4,907 (Pump, $n = 4$).

Effect of transfusion technique on RBC half-life

After 24 hours, there was no significant difference in the circulating half-life of red cells when administered by pump method when compared to the gravity control, i.e. those that survived the first 24 hours showed no subsequent change in survival time (Figure 2). Two-way ANOVA demonstrated that red cell count declined significantly with time ($P < 0.001$), as expected, while method of delivery had no significant effect ($P = 0.619$), and there was no interaction between time and delivery technique (i.e. the effect of time was the same for both techniques). Red cell count declined in a linear manner, with an average observed half-life of 43 days. The single detectable population of red cells delivered by syringe was not detectable at 7 days, and no further analysis of half-life of this population was carried out.

Effect of transfusion technique on RBC counts, plasma hemoglobin and osmotic fragility

No significant differences in RBC count or hemoglobin concentration due to the 3 methods were detected (P values were 0.059 and 0.596, respectively, Figure 3). Post-hoc analysis of RBC count using Tukey's multiple comparison test detected a significant difference between the syringe- and pump-administered groups, with syringe-administered cells having a higher mean RBC count than the pump-administered cells. Neither of these groups, however, was significantly different from the gravity-administered control group. Two-way analysis of variance of osmotic fragility data demonstrated a significant effect of saline concentration on percentage hemolysis, as expected. No significant influence of transfusion method on osmotic fragility was detected ($P = 0.733$, Figure 4).

Discussion

The data presented here suggests that the method of transfusion used to deliver autologous red blood cells has a substantial effect on probability of short-term survival of the transfused cells. Particularly remarkable is the apparent effect of delivery via syringe pump and microaggregate filter (see Table 1), where only 1/7 possible red cell preparations yielded detectable cell populations the day following transfusion. This single viable population of cells was no longer detectable at 7 days following transfusion. By comparison the delivery of autologous red blood cells using a volumetric pump was associated with a 50% probability of loss of the transfused cells by the day following transfusion, while cells transfused using the gravity flow (control preparations) were all detectable the day following transfusion, and demonstrated good long-term survival (Figure 2). Our initial hypothesis was that the method of transfusion would have an effect on the lifespan of transfused autologous canine erythrocytes, however we rejected this hypothesis on observation that the transfused cells, having survived the initial equilibration period, showed no significant difference in circulating half-life over the following sampling period (Figure 2). Volumetric recovery of the RBC's delivered by volumetric pump that survived to sampling was no different to control.

The reasons for the dramatic losses of autologous RBCs following both pump and syringe administration methods are not immediately apparent. As measured differences in osmotic fragility, red cell count and free hemoglobin were not significant when compared to the gravity-administered controls, it is probable that some process is occurring *in vivo* within the first 24 hours, leading to their destruction. With respect to the syringe pump

delivery method, we speculate that shearing stresses resulting from forcing the blood through the microaggregate filter may result in sufficient minor damage to the red cells, that they are removed by the reticuloendothelial system. Other investigators have reported *in vitro* studies that demonstrate that RBC hemolysis is significantly affected by the pump type, age of the transfused blood unit, and the presence of in-line filters.¹⁶⁻¹⁸ Interestingly, lower flow rates induce greater damage to RBCs, while needle gauge, tubing length and tubing diameter had no effect.¹⁷ In the present study, autologous RBCs were administered at the maximum rate commonly used in our institution, thus it is possible that greater damage to red cell populations may have occurred if lower infusion rates (such as commonly used when starting the transfusion process) were utilized.

Two of the eight syringe-administered preparations could not successfully be transfused due to coagulation of the labeled RBCs when mixed with autologous plasma. The plasma had been refrigerated during storage, and we speculate that the mixing of room temperature RBCs with the cold plasma triggered this clotting. We have not, however, further tested this hypothesis. The possible presence of microclots or activation of coagulation factors in other RBC populations administered by the syringe pump method can not be ruled out. Indeed, 1 possible explanation for the unexpected severe losses of RBC's delivered using the microaggregate filter and syringe pump in this study could be more severe shear stresses than typically encountered while using these devices if large numbers of microclots were present in the delivered cells, as this would be expected to increase the pressure and hence shearing stress necessary to deliver the blood product through the filter. This effect would not have been replicated in our *in vivo* studies as the

cells in those studies were not subjected to cold plasma addition or labeling reactions prior to passage through the microaggregate filter.

Age-dependent clearance of red blood cells from circulation is thought to be facilitated by the denaturation of hemoglobin late in the life of the red cell.¹⁹ This oxidation of hemoglobin induces clustering of the integral membrane protein, band 3. Band 3 clustering creates an epitope on the cell surface allowing autologous IgG binding and thus phagocytosis by macrophages. At present this is the best hypothesis to explain the recognition and removal of senescent red cells. It is possible that mechanical trauma to the red cells induced by the volumetric or syringe pumps could have caused an increase in the denaturation/oxidation of hemoglobin, and thus increased removal. Luten et al,¹⁸ hypothesized that the fraction that is removed in the first 24 hours after a transfusion probably consists of irreversibly damaged RBCs.¹⁸

The average observed half-life of labeled RBC's in this study was 43 days. This is lower than values previously reported for non-Greyhound dogs (104 ± 2.2 days) using biotin-labeled RBCs.¹⁵ It is important to acknowledge some aspects of the present study that may have altered red cell survival independent of the transfusion method used. The degree of processing required to add biotin to the cells is considerably greater than that involved in normal processing of a donor blood unit. This extra processing of the cells may have increased their fragility, such that unlabeled populations of red cells may not demonstrate the same survival curves per treatment as the populations in our study. We also speculate that the extra cell handling may have resulted in shorter overall half-life of all cells, including controls, and this may account for the shorter half-life determined for control cells in this study when compared to the half-life reported by other investigators.¹⁵

To assess red cell survival in the present study, biotin labeling of specific red cell populations was carried out followed by quantitative flow cytometry. Biotin labeling has been used successfully to determine blood volume and also to assess *in vivo* survival of RBCs.²⁰⁻²² Biotin labeling has been demonstrated to be an effective and safe method of marking red blood cells for sampling over time. There is evidence of the formation of antibodies to the biotin present on RBCs, however the significance of this in regards to survival of the red cell and detriment to the patient is unknown.²³ Christian et al,²⁴ found that senescence of canine biotinylated red blood cells was characterized by the binding of autologous anti IgG, and that antibiotin antibodies did not play a role.²⁴ Recent studies have demonstrated that it is possible to label and detect up to 5 populations of RBCs within an individual, enabling distinct experimental populations to be tracked over time in the same individual.²⁵ In the study described here we have utilized a similar biotinylation method to identify 3 distinct populations of transfused autologous red cells using flow cytometry, thus allowing us to track survival of populations delivered with differing techniques within the same individual.

The present study utilized a comparatively small number of healthy animals, thus the power of this study to detect significant differences in half-life may be reduced. The present study, however, did identify a substantially altered probability of transfusion survival in the immediate post-transfusion period. Of the 3 methods assessed in this study, the greatest effect was noted when red cells were transfused using a syringe pump and microaggregate filter. This method of blood administration is more commonly used in smaller patients (eg, cats, very small dogs), and replication of this finding in cats is warranted.

The clinical importance of the findings reported here are difficult to accurately assess. Anecdotally, some practitioners observe that the increased PCV following some transfusions does not appear to last as long as expected, and the phenomenon responsible for loss of autologous RBCs in the present study may be involved in the clinical observation of “lost” transfusions, however in those cases the presence of the disease that prompted the initial need for transfusion can not be ignored.

Only 1 pump type^d was used in the present study. This pump was chosen due to the common use of this model in veterinary practices, however it is not the only model of pump potentially used to transfuse RBCs, and other pumps using other methods for delivery could well have different effects on delivered RBCs. Based on the findings of the present study, the authors do not recommend the use of this particular pump for administration of transfusions, however a complete cessation of all pump usage for delivery of RBCs is not warranted on the basis of the data presented here. Additional factors, such as the substantial handling of RBCs imposed by our study design and the viscosity differences between whole blood and packed RBCs that may also have influenced our findings. Although findings of the present study are compelling, the authors urge that careful consideration should be undertaken before substantive changes in clinical approaches to transfusion medicine are made.

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Footnotes:

^a Baxter Healthcare, Deerfield, IL

^b Calbiochem/EMD Chemicals, Gibbstown, NJ

^c Animal Blood Bank, Dixon, CA

^d Flo-Gard 6200, Baxter Healthcare, Deerfield, IL

^e AS50, Baxter Healthcare, Deerfield, IL

^f Hemonate filter, Utah Medical Products, Midvale, UT

^g Thermo Scientific, Rockford, IL

^h VWR Model 1570 Bench Top Agitator, VWR, Bridgeport, NJ

ⁱ Becton-Coulter FC500 Flow Cytometer, Beckman-Coulter Inc, Miami, FL

^j Advia 120, Siemens Healthcare Diagnostics, Deerfield, IL

^k ND-1000 Spectrophotometer, NanoDrop Products, Wilmington, DE

^l The 'R' Statistical Programming Environment (<http://www.r-project.org/>)

^m GraphPad Prism 5.0, Graphpad Software, Menlo Park, CA

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Table 1 – Contingency table showing overnight survival of biotin-labeled, autologous red blood cell populations following transfusion using one of three delivery techniques. The probability of detection of both volumetric pump- and syringe-delivered populations was significantly reduced compared to gravity flow control ($p < 0.001$ for both, Fischer’s Exact Test).

Delivery Technique	Detected	Not Detected	Expected
Gravity Flow	8	0	8
Volumetric Pump	4	4	8
Syringe Pump	1	6	7

Figure Legends

Figure 1 – Representative flow-cytometry results from a dog one day following autologous transfusion with three separate populations of biotin-labeled red blood cells. The gates (f,g,h) represent the expected locations of the three populations administered. This dog shows only the low-label population, administered by gravity flow in this dog.

Figure 2 – Decrease over time in labeled red cell populations (relative to 100% at day 1) following transfusion via gravity flow (n=8) or volumetric pump (n=4). Lines show a linear regression line, while ribbons show the 95% confidence interval of the regression lines. There is a significant decline in both populations over time, the populations show no differences due to transfusion technique.

Figure 3 – Box and whisker plots of total red cell count (left) and plasma hemoglobin (right) following exposure of canine whole blood to gravity flow, volumetric pump or syringe pump + aggregate filter (n=7/treatment). There are no significant differences overall (one-way ANOVA, p=0.059 and 0.596 respectively). Post-hoc analysis shows a significant difference between pump treated and syringe treated red blood cell counts, however neither group differs significantly from control.

Figure 4 – Osmotic fragility curves from canine blood samples (n=7/treatment) following exposure to gravity flow (Control), volumetric pump (Pump) or syringe pump + aggregate filter (Syringe). Total hemolysis (100%) is assumed at a saline concentration of 0.0%. Lines

represent a local least-squares regression line, while ribbons show the 95% confidence interval of the regression lines. The populations show no differences due to transfusion technique.