J Vet Intern Med 2010;24:1131–1137

Effect of Leukoreduction on Transfusion-Induced Inflammation in Dogs

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Background: Removal of leukocytes (LR) has been shown to eliminate or attenuate many of the adverse effects of transfusion in experimental animals and humans.

Hypothesis/Objectives: Transfusion of stored packed red blood cells (pRBCs) is associated with an inflammatory response in dogs and prestorage LR attenuates the inflammatory response.

Animals: Thirteen random-source, clinically healthy, medium and large breed dogs.

Methods: Experimental study. On day 0, animals were examined and baseline blood samples were collected for analysis. Whole blood was then collected for processing with and without LR, and stored as pRBC. Twenty-one days later, stored pRBCs were transfused back to the donor. Blood samples were collected before and 1 and 3 days after transfusion.

Results: In the dogs that received non-LR pRBCs (n = 6) there was a significant increase from baseline in white blood cell count from a mean (SD) of 8.20 (2.74) to 13.95 (4.60) $\times$ 10^3 cells/μL ($P < .001$) and in segmented neutrophil count from a mean (SD) of 5.76 (2.70) to 11.91 (4.71) $\times$ 10^3 cells/μL ($P < .001$). There were also significant increases in fibrinogen from a mean (SD) of 129.7 (24.2) to 268.6 (46.7) mg/dL ($P < .001$) and C-reactive protein from a mean (SD) of 1.9 (2.1) to 78.3 (39.3) μg/mL ($P < .001$). There was no significant increase from baseline in any of the markers in the dogs that received LR pRBC (n = 5).

Conclusions and Clinical Importance: There is a profound inflammatory response to transfusion in normal dogs, which is eliminated by LR of the pRBC units.

Key words: Blood banking; C-reactive protein; Fibrinogen; Leukocytes.

Blood transfusion is an important part of the treatment of many medical and surgical diseases of companion animals, but there are numerous complications associated with the administration of blood products. Fortunately, overt transfusion reactions in dogs are relatively infrequent, with incidence rates of 3.0, 3.3, 4.2, and 13%.[1–4]

Although the frequency of overt adverse reactions to blood transfusions in dogs appears to be relatively low, it is possible that a clinically silent inflammatory response to blood transfusion might be overlooked, because many transfusion recipients have ongoing inflammation associated with the underlying illness. It is also likely that any possible negative impact of transfusion on the status of a critically ill dog would be underestimated or attributed to the primary disease process. The fatality rates of dogs undergoing transfusion has been reported to range from 39 to 53%, with most deaths attributed to the underlying disease process.[2,4]

The most common adverse consequences of compatible blood transfusion are febrile nonhemolytic reactions, immune suppression, decreased platelet counts, acute lung injury, and urticaria.[5] Blood transfusions in humans are associated with an inflammatory response.[6,7] White blood cells (WBCs) in transfused products are the cause of the febrile reactions because of WBC cytokine production in stored products, immune suppression via decreases in natural killer cell function, phagocytosis, and decreased helper to suppressor cell ratios, decreased platelet counts via alloimmunization, and acute lung injury via WBC aggregates in the pulmonary circulation.[5,8] Contaminating leukocytes in packed red blood cell (pRBC) transfusions can cause immunosuppression via down regulation of natural killer cell activity and T-cell proliferation.[9] Leukocyte lysis during storage releases immunomodulators such as histamine, myeloperoxidase, plasminogen activator inhibitor-1, and eosinophilic cationic protein.[10] Leukoreduction (LR) attenuates or eliminates the inflammatory response to blood transfusion in humans.[11,12]

We hypothesized that transfusion of stored pRBCs is associated with a clinically silent inflammatory response in dogs as it is in humans. We further hypothesized that LR of blood before storage would attenuate the expected inflammatory response.

Abbreviations:

LR	leukoreduction
pRBCs	packed red blood cells
WBC	white blood cell

Materials and Methods

Dogs

The study population consisted of 13 random-source, medium and large breed dogs (9 intact females, 4 intact males, weight range 19.5–32 kg). Results of a complete blood count, biochemistry profile, and physical examination were normal for each dog. Animals

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Submitted October 1, 2009; Revised March 19, 2010; Accepted May 11, 2010.

10.1111/j.1939-1676.2010.0561.x

were randomly assigned to 2 groups to receive either non-LR blood (n = 7) or LR blood (n = 6). All dogs were maintained in Association for Assessment and Accreditation of Laboratory Animal Care–approved housing and cared for according to the principles outlined in the NIH Guide for Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Care and Use Committee.

Study Schedule

On day 0, animals were examined and blood samples were collected for analysis (baseline = B), followed by collection of 376–392 mL of whole blood for processing and storage of pRBCs. The fresh plasma was returned to the donor and the pRBCs were stored for later use. Blood samples were again collected from the dogs for analysis 21 days later (pretransfusion = T0), after which the stored pRBCs were transfused into the donor. On day 22 (1 day posttransfusion = T1) and day 24 (3 days posttransfusion = T3), blood samples were again collected for analysis. One dog from each group was excluded because of clinically important transfusion reactions, bringing the total number used for analysis to 11 (LR n = 5, non-LR n = 6). Because of an error, citrated plasma samples were not available for the non-LR group at the time T3, so fibrinogen concentration could not be measured. On days T1 and T3, dogs were not examined for clinical evidence of inflammation.

Collection and Handling of Blood for Transfusion

Animals were sedated on day 0 with 0.01 mg/kg atropine, 2–3 μg/kg dexmedetomidine, and 0.05 mg/kg butorphanol IM. Once the sedation had taken effect, a peripheral catheter was placed. Whole blood was collected by aseptic technique via atraumatic jugular venipuncture into either a conventional triple bag system with 100 mL of adsol preservation solution[a] (n = 7) or a whole blood integral filter blood container system[b] (n = 6). The sedation was reversed with atipamezole (0.01 mg/kg IM) as soon as blood collection was complete, and lactated Ringer's solution (250 mL) was infused through the peripheral catheter to partially replace the blood volume lost.

For units collected into LR filter bags, the whole blood was stored before filtration at 4°C for 4 hours as described previously.[13,14] The full bag was weighed before separation of the components. To perform filtration, the integral cannula was broken allowing the blood to flow through the in-line filter. Units were hung with the tubing extended to allow filtration to occur via gravity flow and filtered at room temperature. Filtration was timed from the start of the blood flow through the tubing to cessation of blood flow. The bags were weighed postfiltration. An aliquot (3 mL) was collected from the prefiltration and postfiltration tubing for evaluation of hematocrit, platelet and WBC counts. The pre- and postfiltration weights were converted to millimeters by dividing the gram weight by 1.06.

Whole blood units (both non-LR and LR) were separated via centrifugation at 5010 × *g* for 15 minutes at 10°C. After centrifugation, plasma was expressed into an additional, attached, storage bag while 100 mL adsol was added to the pRBCs from the satellite bag. The fresh plasma was returned to the donors over approximately 1 hour to replace volume and plasma proteins lost. The pRBCs were stored upright at 4°C and gently mixed every 2 days for 21 days.

Transfusions

On day 21, after collection of T0 blood samples, a catheter was aseptically placed in the cephalic vein. The dogs then received a transfusion of their own stored pRBCs over a period of 2 hours. Dogs were examined and vital signs evaluated before transfusion, every 15 minutes during the 1st hour of the transfusion, and at completion of the infusion, but not on the following days.

Blood Collection and Handling for Analysis

Blood was collected via venipuncture from a jugular vein with a 21 G collection set with a vacutainer adaptor. Samples were collected directly into vacutainer tubes. Order of sample collection was nonactivated sample (red top) for production of serum, EDTA sample, then citrated sample. Citrated samples were collected at 9 : 1 ratio (blood : citrate) into 3.2% citrate in siliconized tubes, then mixed thoroughly by repeated inversion.

EDTA blood was immediately submitted for cell counts. Citrated blood was immediately centrifuged at 3000 × *g* for 10 minutes. Plasma was removed and aliquotted, flash frozen on 100% ethanol with dry ice, then stored at −80°C until analysis. For serum production, blood in red top tubes was allowed to clot at room temperature for 1 hour, then centrifuged at 3000 × *g* for 10 minutes. Serum was removed, aliquotted and frozen at −80°C until analysis.

Blood and Plasma Analysis

Cell counts on EDTA blood and on samples from the whole blood units (pre- and postfiltration) were performed by the VTH clinical laboratory on a Cell-dyne 3700 hematology analyzer. Plasma fibrinogen concentration was measured in the stored citrated plasma by the Clauss method[c] on a mechanical coagulometer.[d] C-reactive protein concentration on stored serum were measured by batch analysis according to the manufacturer's directions with a commercially available previously validated[15] ELISA kit.[e]

Statistical Analysis

Statistical comparisons were performed by SigmaStat 2.03. Data were compared by a two-way repeated measures ANOVA with assessment for effects of both time point and treatment group as factors. When the ANOVA identified statistically significant differences, a Tukey test was used for multiple comparison analysis to isolate the factors that differed. Characteristics of the blood units were compared by a *t*-test. Significance was defined at a P value of $< .05$.

Results

Animal Observations

No adverse events were noted during the sedation and collection portions of the study. No dogs developed clinical signs of transfusion reactions (including changes in heart rate, respiratory rate, or rectal temperature) during the pRBC infusion. One dog in each group developed clinically apparent transfusion reactions after completion of the infusion. One dog that received non-LR pRBCs had evidence of slight hemoglobinuria 4 hours after transfusion, and moderate hemoglobinemia and marked hemoglobinuria 20 hours after transfusion with mild increases in hepatic enzymes. One dog that received LR pRBCs developed transient fever, malaise, and vomiting after completion of the infusion and lasting approximately 4 hours. Because clinically apparent transfusion reactions would be expected to be associated with a profound inflammatory response, results from both of these animals were excluded from statistical evaluations of WBC, fibrinogen, and C-reactive protein data.

Characteristics of the Collected Units

There was no difference in mean (SD) blood volume collected between the two types of units [non-LR 376 (33) mL; LR 392 (69) mL] or in the volume of adsol-preserved pRBCs obtained [non-LR 336 (40) mL; LR 346 (41) mL].

The filtration procedure lasted a mean (SD) of 25.7 (5.0) minutes. The mean (SD) amount of blood lost to the filter was 49.8 (6.7) mL. Essentially all leukocytes and platelets were removed by the filter. Mean (SD) prefiltration and postfiltration WBC counts on the units were 7,230 (3,197) and 13 (8) cells/μL, respectively, resulting in mean reduction of 99.79%. Mean (SD) prefiltration and postfiltration platelet counts on the units were 270,200 (47,300) and 39 (61) cells/μL, respectively, resulting in mean reduction of 99.98%. Mean (SD) RBC count was only slightly decreased by filtration [pre: 6.56 (0.59); post: 6.35 (0.56) $\times$ 10^6 cells/μL] with a mean recovery of 96.8%.

Effects of Transfusions on Indicators of Inflammation

Total WBC counts and segmented neutrophil counts were significantly different when evaluated for effects of either time or group, with a significant interaction between time point and group. For dogs (n = 6) receiving non-LR units, total WBC counts (reference range: 6,000–17,000 cells/μL) and segmented neutrophil counts (reference range: 3,000–11,500 cells/μL) were significantly increased on the day after transfusion (T1) compared with both pretransfusion time points (B, T0), but were not different from pretransfusion values by T3 (Figs 1A and 2A). For dogs receiving LR units (n = 5), total WBC counts and segmented neutrophil counts were not different when compared between all time points (Figs 1B and 2B). Comparison of WBC and segmented neutrophil counts after transfusion (at T1) between dogs receiving non-LR and LR units indicated significantly lower counts in dogs receiving LR units. WBC types other than neutrophils were unchanged at T1 as compared with pretransfusion time points in both groups (data not shown).

Fibrinogen concentration (reference range: 107–244 mg/dL) was also significantly different when evaluated for effects of either time or group, with a significant reaction between time point and group. For dogs receiving non-LR units, plasma fibrinogen concentration was significantly increased on the day after transfusion (T1) compared with both pretransfusion time points (B, T0) (Fig 3A). For dogs that received LR units, fibrinogen concentrations at T1 were not different from either pretransfusion time points (B, T0), although they were increased slightly at T3 compared with B (Fig 3B). Comparisons by time point between the non-LR and the LR groups indicated significant difference only for the T1 time point, with the LR group having lower fibrinogen concentration.

Similarly, serum C-reactive protein (reference range: 0–4.7 μg/mL) was significantly different when evaluated for effects of either time or group, with a significant interaction between time point and group. Serum C-reactive protein concentration was significantly increased on the day after transfusion (T1) compared with both pretransfusion time points (B, T0) in dogs receiving non-LR units. C-reactive protein concentration had significantly decreased by T3 (Fig 4A). In dogs receiving LR units, serum C-reactive protein concentration was not significantly different between any time points (Fig 4B). Comparisons by time point between the non-LR and the LR groups indicated significant difference for the T1 and T3 time points.

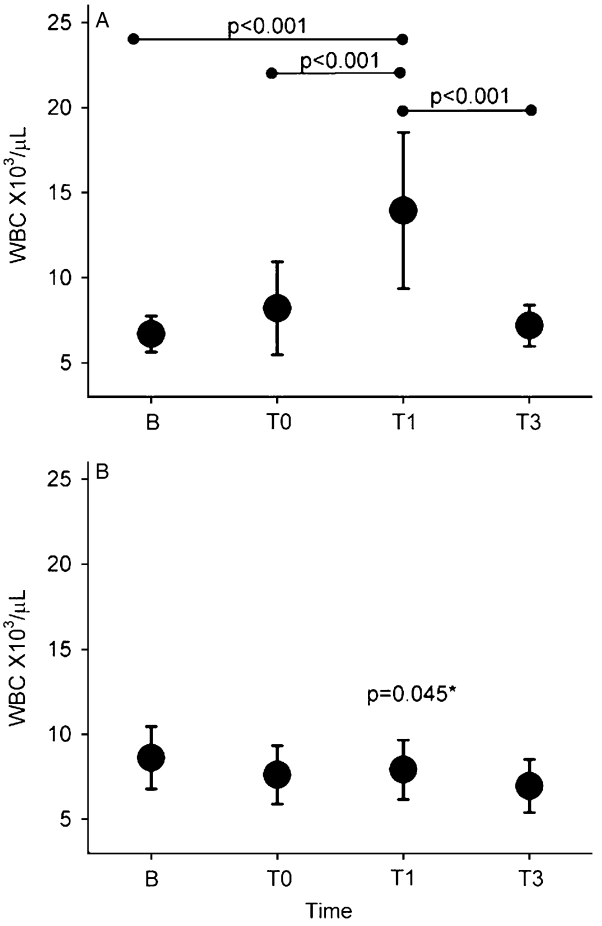

Fig 1. Total leukocyte counts. Total white blood cell (WBC) counts in dogs receiving nonleukoreduced (non-LR, **A**) or leukoreduced (LR, **B**) packed red blood cell units. For multiple pairwise comparisons that achieved statistical significance, *P* values are reported with associated lines indicating which time points were compared. If no line and value are reported, the comparison did not achieve significance. **P* values are comparisons between non-LR and LR groups at each time point. Data points reported are mean values, with error bars representing standard deviation. B, baseline (day 0); T0, pretransfusion (day 21); T1, 1 posttransfusion (day 22); T3, 3 days posttransfusion (day 24).

Discussion

The results obtained in this study provide evidence that transfusion of pRBCs is associated with a significant inflammatory response in normal dogs. The inflammatory response is clearly indicated by the marked increases

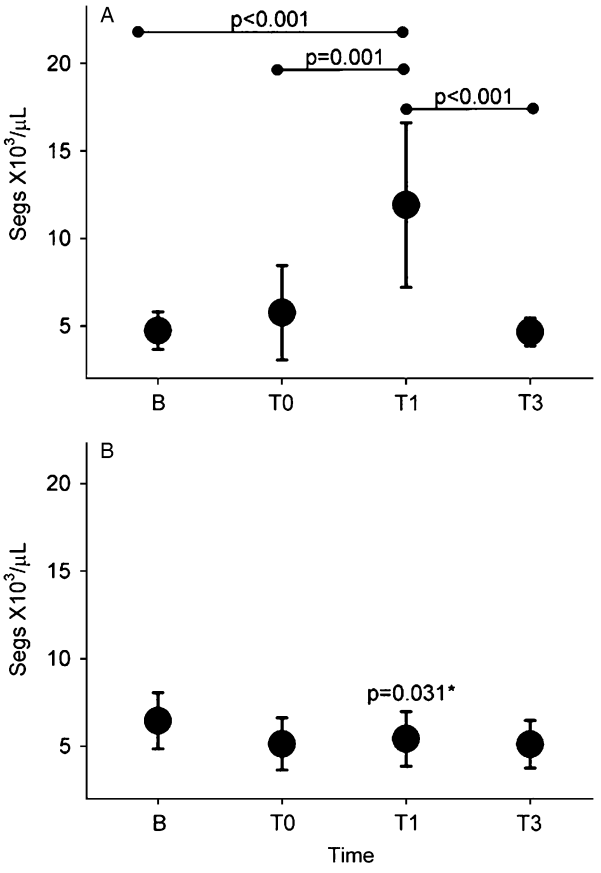

Fig 2. Segmented neutrophil counts. Segmented neutrophil (Segs) counts in dogs receiving nonleukoreduced (non-LR, **A**) or leukoreduced (LR, **B**) packed red blood cell units. For multiple pairwise comparisons that achieved statistical significance, *P* values are reported with associated lines indicating which time points were compared. If no line and value are reported, the comparison did not achieve significance. **P* values are for comparisons between non-LR and LR groups at each time point. Data points reported are mean values, with error bars representing standard deviation. B, baseline (day 0); T0, pretransfusion (day 21); T1, 1 posttransfusion (day 22); T3, 3 days posttransfusion (day 24).

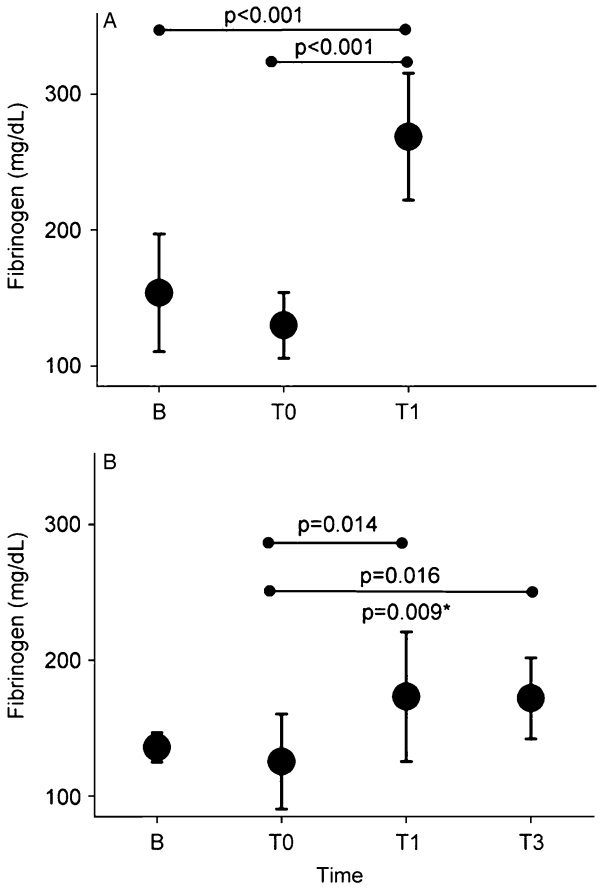

Fig 3. Plasma fibrinogen concentration. Plasma fibrinogen concentrations in dogs receiving nonleukoreduced (non-LR, **A**) or leukoreduced (LR, **B**) packed red blood cell units. For multiple pairwise comparisons that achieved statistical significance, *P* values are reported with associated lines indicating which time points were compared. If no line and value are reported, the comparison did not achieve significance. **P* values are for comparisons between non-LR and LR groups at each time point. Data points reported are mean values, with error bars representing standard deviation. B, baseline (day 0); T0, pretransfusion (day 21); T1, 1 posttransfusion (day 22); T3, 3 days posttransfusion (day 24). Because of an error, samples were not available for the non-LR group on day T3 for measurement of fibrinogen.

in total leukocyte counts (of approximately 2-fold primarily because of an increase in circulating segmented neutrophils), fibrinogen level (of approximately 2-fold), and C-reactive protein (of approximately 60-fold). Increases in these markers of inflammation were apparent only in the group receiving pRBC units produced through standard blood banking methods (without LR). The increases in all parameters in response to the transfusion of non-LR blood were markedly greater in magnitude than the normal day-to-day variation (as indicated by comparison of time points B and T0). Further, the C-reactive protein concentration observed in response to transfusion of non-LR blood were comparable to those reported for clinical canine patients with sepsis,[16,17] pancreatitis,[16] trauma,[16] meningitis,[18] pyometra,[19] babesiosis,[20] or inflammatory bowel disease.[21]

Several studies in humans have described an inflammatory response to transfusion similar to that observed in our normal dogs. There is an immediate 60% increase in WBC count after transfusion in human patients that persists at 12 hours posttransfusion, but had returned to baseline at 24 hours.[22] Another study documented posttransfusion leukocytosis in sick preterm neonates.[23] Among human patients undergoing cardiac surgery, those receiving transfusions had significantly more profound release of inflammatory mediators than those not receiving transfusion.[24] In an in vitro study a significant increase in 7 markers of inflammation occurred during storage of non-LR packed RBCs with no significant increase seen in LR blood.[7]

Our study did not address the question of the mechanism underlying this inflammatory response. The observed inflammatory response could not have been because of antibodies against erythrocyte surface or other cell antigens, because each animal received their own

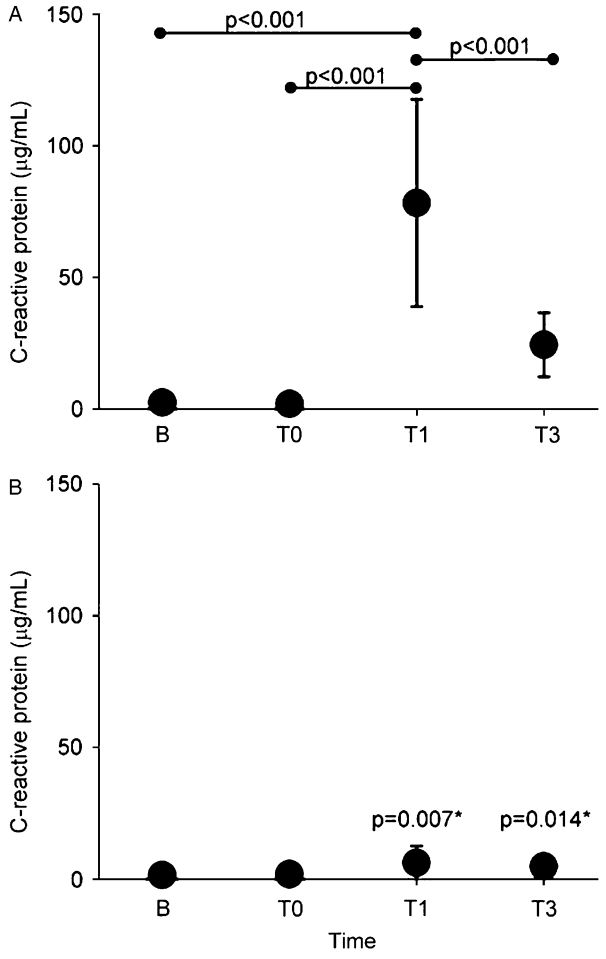

Fig 4. Serum C-reactive protein concentration. Serum C-reactive protein concentrations in dogs receiving nonleukoreduced (non-LR, **A**) or leukoreduced (LR, **B**) packed red blood cell units. For multiple pairwise comparisons that achieved statistical significance, *P* values are reported with associated lines indicating which time points were compared. If no line and value are reported, the comparison did not achieve significance. **P* values are for comparisons between non-LR and LR groups at each time point. Data points reported are mean values, with error bars representing standard deviation. B, baseline (day 0); T0, pretransfusion (day 21); T1, 1 posttransfusion (day 22); T3, 3 days posttransfusion (day 24).

cells. In humans, febrile nonhemolytic reactions to nonleukoreduced blood may occur via several mechanisms: (1) immune recognition of donor leukocytes by recipient via anti-leukocyte antibodies; (2) immune destruction of incompatible donor platelets by recipient antibodies; and (3) passive transfer of donor inflammatory cytokines.[12] Only the latter mechanism could have been responsible for the observed inflammatory response in our normal dogs that received non-LR blood, because recipients received their own donated cells. Removal of the WBCs and platelets before storage of units with commercially available in-line LR filters prevented the inflammatory response to transfusion, supporting the hypothesis that the observed changes in markers of inflammation was associated with contamination of the stored RBCs with either leukocytes, platelets, or both.

The role of the length of blood storage in production of an inflammatory response in the recipient is not yet clear. In the current study, the pRBC units were stored for 21 days before administration, which is well within current guidelines for appropriately preserved RBC storage. It is possible that markedly limiting the storage time could reduce cytokine production by contaminating leukocytes in the unit. However, recipients receiving fresh pRBCs would still be exposed to donor WBCs and platelets, and could still produce immune responses to incompatible donor cells. Furthermore, changes in animal blood banking methods to drastically limit storage time would be extremely impractical as a blood banking procedure.

Regardless of the underlying mechanism or relationship to storage time, administration of a therapeutic agent that causes an inflammatory response is of real concern for the patient population likely to receive blood transfusion. Patients requiring RBC support are often seriously ill with diseases already associated with a marked inflammatory response. In particular, dogs with immune-mediated hemolytic anemia, neoplasia, and sepsis are generally experiencing profound inflammatory responses because of the underlying illness. These inflammatory processes are associated with significant morbidity and serious sequelae, including but not limited to oxidative stress, disseminated intravascular coagulation, thrombosis-thromboembolism, hypotension, and organ failure. Studies in humans have suggested adverse consequences for patients receiving transfusions. One study reported that blood transfusion was a significant independent predictor of systemic inflammatory response syndrome, intensive care unit admission, and mortality in trauma patients.[25] Clinically, an increase in WBC count or other indicators of inflammation in response to transfusion might be inappropriately ascribed to other changes in patient status such as infection, worsening inflammatory disease, or postoperative inflammation. Regardless, direct administration of an inflammatory stimulus via transfusion has the potential to adversely impact already compromised patients, and may therefore be inappropriate.

LR is standard of practice in human blood banking in Canada and much of Europe because it has been clearly documented to reduce the incidence of transfusion reactions and inflammatory responses in recipients.[12,26] A decreased incidence of febrile, nonhemolytic transfusion reactions was seen in patients receiving multiple transfusions (61% with nonfiltered blood versus 2.5% with prestorage LR filtered blood).[27] In a critically ill population, patients receiving non-LR pRBCs had a 44% increase in WBC count, as compared with a significantly lower increase in WBC count of 10.1% among patients receiving LR blood.[6] Prestorage filters decreased reactive oxygen species and myeloperoxidase produced by intact leukocytes in stored blood,[7,28] and showed superior endothelial cell function in a canine model of ischemia reperfusion injury compared with non-LR blood.[29]

Other potential benefits of prestorage LR include better RBC survival, reduction of posttransfusion immunosuppression, and reduction of transfusion-associated transmission of infection. Leukocytes are thought to contribute to decreased RBC survival via

depletion of glucose and direct effects of cytokines on RBCs.[30] Studies have shown that adenosine triphosphate is better preserved in LR RBC components.[30] A decreased incidence of postoperative infections has been reported in patients receiving a transfusion of LR RBCs compared with patients receiving non-LR blood.[31] Postoperative infection developed in 13 patients (23%) receiving non-LR blood but in only 1 patient (2%) that received LR blood.[31] Canine renal allograft survival increased after blood transfusion which was suspected to be because of immunosuppression caused by the transfusion.[32] LR has also been shown to decrease the tumor growth that conventional blood transfusion appears to support.[33] Additionally, LR filters may remove 75–100% of contaminating bacteria.[34] In one study, blood units inoculated with bacteria were subjected to LR, then evaluated via culture after 42 days of storage. The positive culture rate was much lower for LR (8%) as compared with non-LR (67%) stored blood.[35] In a meta-analysis of 9 clinical trials and 11 meta-analyses concluded that LR significantly reduced the odds of postoperative infection by approximately 50%.[36]

There is currently a debate over mandatory LR in the United States because some studies have failed to demonstrate a clear benefit of such a practice. In a randomized, double-blinded clinical trial of 268 trauma patients no difference was found in infectious complications in the LR group compared with the non-LR group.[37] In a secondary analysis of that same study there was no difference in the incidence of pulmonary complications (acute lung injury, acute respiratory distress syndrome) in either group.[38]

Use of LR blood in a clinical setting for canine transfusion has not been described. However, the successful removal of WBCs from canine blood with in-line LR filters, and the lack of relevant impact of LR on RBC viability, has been reported previously.[13] We confirmed the findings of Brownlee and colleagues by demonstrating essentially complete removal of leukocytes and platelets using a 4-hour cooling time before filtration. Note, however, that the cell counting method used was not the optimal approach for measuring very low cell counts and may have overestimated the degree of LR.[39] LR collection sets are only slightly more expensive than standard sets with respect to the total cost of blood transfusion (may add ~$30 to each unit), making them feasible for use in canine blood banking procedures. We demonstrated a profound impact of LR on reduction of the inflammatory response to transfusion in normal dogs. Future studies on the effect of LR in clinical patients will help to shed light on the benefit to cost ratio for these patients. If LR reduces or eliminates the inflammatory response in clinical patients as well, this approach could improve the safety of transfusion therapy in veterinary medicine.

Footnotes

[a] Adsol, Fenwal Laboratories, Baxter Healthcare Corp, Deerfield, IL

[b] Sepacell RS2000, Baxter Healthcare Corp

[c] Fibri-prest Automate 2, Diagnostica Stago, Asnieres, France using purified human fibrinogen (Enzyme Research Laboratories [Burlington, VT] as a standard)

[d] ST4 Coagulometer, Diagnostica Stago, Asnieres, France

[e] Phase C-reactive Protein, Tridelta Development Ltd, Kildare, Ireland

Acknowledgments

We thank Rhiannon Ardisana and Ashley Freeland for their assistance with blood collection, and Jessica Garrett for her assistance with blood unit centrifugation, and Sandra Rodriguez-Zas for statistical advice.

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