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

S.M. Muro, J.H. Lee, J.V. Stokes, M.K. Ross, T.M. Archer, R.W. Wills, A.J. Mackin, and J.M. Thomason

Background: Storage of canine packed red blood cells (pRBCs) can increase erythrocyte phosphatidylserine (PS) expression and eicosanoid concentrations.

Hypothesis/Objectives: To determine the effects of leukoreduction on erythrocyte PS expression and eicosanoid concentrations in stored units of canine pRBCs. Our hypothesis was that leukoreduction would decrease PS expression and eicosanoid concentrations.

Animals: Eight healthy dogs.

Methods: In a cross-over study, units of whole blood were leukoreduced (LR) or non-LR and stored (10 and 21 days) as pRBCs. Samples were collected at donation, and before and after a simulated transfusion. PS expression was measured by flow cytometry, and concentrations of arachidonic acid (AA), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), thromboxane B_2 (TXB_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and leukotriene B_4 (LTB_4) were quantified by liquid chromatography–mass spectrometry.

Results: There was no change in PS expression during leukoreduction, storage, and simulated transfusion for non-LR and LR units. Immediately after leukoreduction, there was a significant increase in TXB_2 and $PGF_{2\alpha}$ concentrations, but during storage, these eicosanoids decreased to non-LR concentrations. In both LR and non-LR units, 6-keto-$PGF_{1\alpha}$ concentrations increased during storage and simulated transfusion, but there was no difference between unit type. There was no difference in AA, LTB_4, PGE_2, and PGD_2 concentrations between unit types.

Conclusions and Clinical Importance: Leukoreduction, storage, and simulated transfusion do not alter erythrocyte PS expression. Leukoreduction causes an immediate increase in concentrations of TXB_2 and $PGF_{2\alpha}$, but concentrations decrease to non-LR concentrations with storage. Leukoreduction does not decrease the accumulation of 6-keto-$PGF_{1\alpha}$ during storage.

Key words: Dog; Prostacyclin; Thromboxane; Transfusion.

Blood transfusions are commonly used for the treatment of anemia in critically ill veterinary patients. Storage of blood products, although necessary, creates an unnatural environment that can lead to accelerated product degradation.[1] In humans and dogs, a direct correlation has been established between blood product storage time and increased morbidity and mortality in transfused patients, suggesting that stored blood products can undergo clinically important changes that lead to increased risk of complications in the recipient.[2–7]

From the Department of Clinical Sciences, (Muro, Archer, Mackin, Thomason); Department of Basic Sciences, (Lee, Stokes, Ross); and the Department of Pathobiology and Population Medicine (Wills), College of Veterinary Medicine, Mississippi State University, Mississippi State, MS.

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Corresponding author: J.M. Thomason, DVM, MS, DACVIM, Department of Clinical Sciences, College of Veterinary Medicine, Mississippi State University, PO Box 6100, Mississippi State, MS 39762-6100; e-mail: thomason@cvm.msstate.edu.

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Abbreviations:

15-PGDH	15-hydroxyprostaglandin dehydrogenase
6-keto-$PGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
AA	arachidonic acid
COX	cyclooxygenase
FACS-PBS	fluorescence-activated cell sorting–phosphate buffered saline
FITC	fluorescein isothiocyanate
LOX	lipoxygenase
LR	leukoreduced
LTB_4	leukotriene B_4
MFI	median fluorescence intensity
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFS	prostaglandin F synthase
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
pRBCs	packed red blood cells
PS	phosphatidylserine
TXB_2	thromboxane B_2

Numerous studies have investigated red blood cell storage lesions, and several mechanisms have been identified as contributors to the degradation of stored erythrocytes.[1,4,8]

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

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

Eicosanoids are synthesized in a wide range of cells, including leukocytes, vascular epithelial cells and platelets. Although erythrocytes contain AA, they do not possess the oxidative enzymes involved in AA metabolism and eicosanoid synthesis.[18] The leukocyte and platelet populations in units of pRBCs probably are the major contributors to AA metabolism.[15] In humans, leukocytes lyse early in storage, releasing enzymes that can damage the erythrocyte, causing an increase in PS expression, and contribute to the synthesis of proinflammatory molecules, including phospholipaseA_2, which can contribute to eicosanoid production.[4,19] With the use of a leukoreduction filter before storage, the majority of leukocytes and platelets are extracted from the unit of blood, preventing these cells from influencing the environment within the pRBC unit, and decreasing the inflammatory response associated with transfusion.[20] In units of stored human pRBCs, the removal of leukocytes and platelets by leukoreduction before storage causes a significant decrease in erythrocyte PS expression.[10] A decrease in the concentrations of leukocytes and platelets in the unit also removes the main source of eicosanoid synthesis.

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

Materials and Methods

Animals

Dogs were chosen from our research colony and included in the study if they were determined to have normal health status and had not been exposed to any medications or vaccines for at least 2 weeks before initiation of the study. Normal health status was established based on normal results of physical examination, CBC, serum biochemistry, urinalysis, and heartworm and tick-borne disease testing. Animal use was approved by the Mississippi State University Institutional Animal Care and Use Committee and was in compliance with the requirements of the American Association for Accreditation of Laboratory Animal Care.

Blood Donation, Leukoreduction, and Sample Collection

Donors were randomly allocated into 1 of 2 groups, a leukoreduced (LR) group and a non-LR group. Each dog underwent a standard blood donation. Briefly, the donors were positioned in either right or left lateral recumbency, and the hair overlying the jugular vein was clipped and the skin aseptically prepared. A 16-gauge needle was inserted into the jugular vein, and approximately 450 mL of blood was collected aseptically, under negative pressure, into a standard triple blood banking bag[a] for the non-LR group and a quadruple blood banking bag[b], containing a leukoreduction filter, for the LR group. The units contained anticoagulant citrate phosphate dextrose solution. No adverse events were detected in the donor dogs during or after blood collection.

For the LR group, the unit was leukoreduced (LR) and platelet-depleted by passage of blood through a leukoreduced filter immediately after collection. To assess the extent of leukoreduction, blood samples were collected before and after leukoreduction from the in-line tubing system for total leukocyte and platelet counts using an automated hematologic analyzer.[c] For both LR and non-LR groups, pRBCs were prepared by separating the red cells and plasma by centrifugation. To remove the plasma after centrifugation, external pressure was applied to the blood bag, and the plasma was passed via a connecting tube into an attached empty bag for storage as fresh frozen plasma. While still containing plasma, the connecting tube between the 2 blood bags was sealed and removed. The plasma in the sealed tube was collected, snap frozen in liquid nitrogen, and stored at −80°C. This plasma represented the initial sample ("donation sample") for eicosanoid analysis. The remaining unit of pRBC was separated into 2 half units by applying external pressure to the unit and allowing erythrocytes to pass, via a connecting tube, into an attached empty bag. While still containing red blood cells, the connecting tube between the 2 blood bags was sealed and used as the initial sample ("donation sample") for determination of PS expression. Each half unit of pRBCs was stored vertically at 4°C in a dedicated refrigerator for 10 or 21 days. The donors underwent at least a 28-day recovery period after blood collection, and then, the groups were switched and the process was repeated.

On day 10 of storage, 1 half unit from each dog (4 LR and 4 non-LR) was removed and samples were collected for analysis. When removed from refrigeration, each half unit was infused with 50 mL of 0.9% saline, mixed gently, and 1 mL of reconstituted pRBCs was removed from the unit for determination of PS expression. An additional 20 mL of reconstituted pRBCs was collected, centrifuged, and the supernatant removed for eicosanoid analysis. This sample was snap frozen in liquid nitrogen and stored at −80°C until analysis. The collected erythrocytes and supernatant represented the "day 10 pretransfusion" samples. To mimic transfusion conditions, the remainder of each half unit was left at room temperature for 5 hours. At the completion of this time period, using the same procedures as mentioned above, a 1 mL sample was collected and processed for the measurement of erythrocyte PS expression, and a 20 mL sample was collected for eicosanoid analysis. The collected erythrocytes and supernatant represented the "day 10 post-transfusion" samples. On day 21 of storage, the 8 half units of pRBCs that had remained in refrigeration were removed and processed similar to the day 10 samples. The erythrocytes and supernatant collected on Day 21 of storage represented the "day 21 pretransfusion" and "day 21 post-transfusion" samples.

Phosphatidylserine Analysis

A flow cytometric assay was used to quantitate the expression of PS on canine erythrocytes. Labeling of cells was performed based on a previously described protocol.[21] Personnel performing flow cytometry was blinded as to whether samples were LR or non-LR. For red blood cell preparation, 50 μL of concentrated erythrocytes was washed twice with fluorescence-activated cell sorting–phosphate buffered saline (FACS-PBS), containing 0.2% bovine serum albumin[d] , and 10 μL of the washed red blood cells was resuspended in 90 μL of FACS-PBS. For flow cytometric analysis, 16 μL of washed RBCs was incubated with annexin-V-fluorescein isothiocyanate (FITC)[e] and annexin binding buffer for 15 minutes in the dark at room temperature. After incubation, 400 μL of annexin binding buffer was added, gently mixed, and analyzed within 1 hour. Flow cytometric analysis was performed using a flow cytometer[f] and computer software[g] . Red blood cell populations were displayed on log forward-scatter versus log side-angle light scatter plots. Gates were adjusted to baseline erythrocyte populations, and 5,000 gated events were recorded for each labeling. For quality control, both positive and negative controls were included. Expression was quantified by the intensity of annexin-V-FITC fluorescence and expressed as median fluorescence intensity (MFI).

Eicosanoid Analysis

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

Statistical Analysis

Sample size calculation was performed based on previously published data.[15] The assumptions used in the calculations were an alpha of 0.05 and a power of 0.95. An estimated sample size of 8 dogs would detect if the concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2 after leukoreduction would be similar to the initial donation sample. A linear mixed model was fit with PROC MIXED in a statistical software program[l] for each outcome. Run, sequence, filter, sample, and filter and sample interaction were included as fixed effects with a Kenward–Rogers degrees of freedom method specified. Dog identity was included as random effect with a variance component covariance structure specified. Repeated measures of dog identity within run for the different samples were specified in a repeated statement with a spatial power law covariance structure. The interaction term was dropped from the model if it was not significant. If the interaction term was significant, differences in least squares means between each of the concentrations of 1 variable were calculated for each concentration of the other variable in the interaction using an LSMESTIMATE statement. The SIMULATE adjustment for multiple comparisons was used for significant effects. The distribution of the conditional residuals was evaluated for each outcome to ensure the assumptions of the statistical model had been met. An alpha level of 0.05 was used to determine statistical significance for all methods.

Results

Animals

Eight healthy adult research Walker Hound dogs, 5 males and 3 females, were used in this study. The mean age of the dogs was 1.5 years (range, 1.5–6.5 years), and their mean body weight was 27.4 kg (range, 20.5–30.5 kg).

Leukoreduction

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

Phosphatidylserine Expression

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

Eicosanoid Concentration

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

At the time of donation, the $PGF_{2\alpha}$ concentrations in LR units were significantly ($P < .0001$) higher than in the non-LR units. The $PGF_{2\alpha}$ concentrations in LR units then decreased rapidly with storage, such that there was no difference in $PGF_{2\alpha}$ concentration between the LR and non-LR units for any of the remaining time

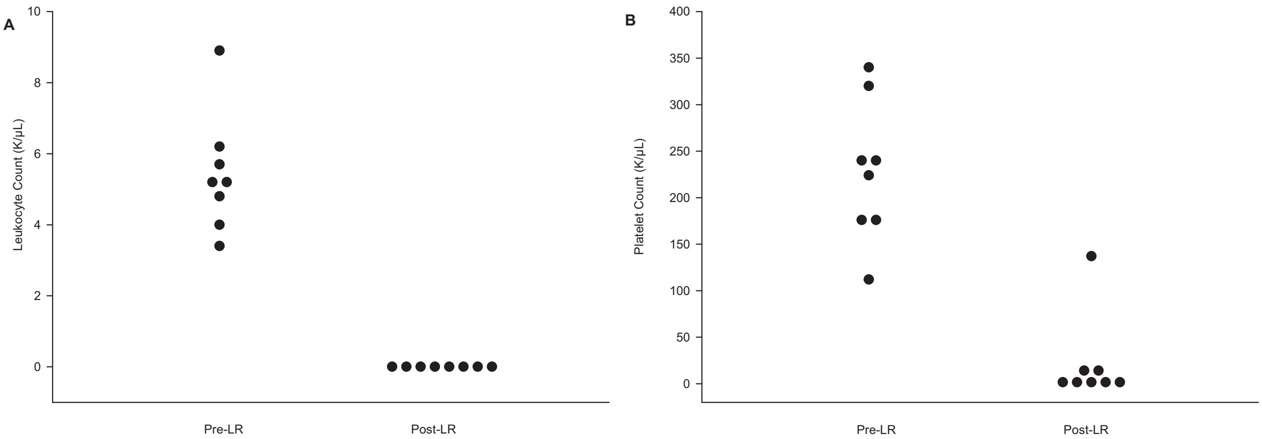

Fig 1. Leukocyte (**A**) and platelet (**B**) counts before and after leukoreduction. LR, leukoreduced.

Table 1. Based on flow cytometric analysis, the MFI (mean ± standard deviation) of canine erythrocyte PS expression, with and without leukoreduction, at collection (donation) and after various lengths of storage (pretransfusion) and after a simulated transfusion (post-transfusion).

Erythrocyte PS Expression (MFI)		
Sample	Non-LR Units of pRBCs	LR Units of pRBCs
Day 0 – Donation	7.74 ± 1.04	7.57 ± 0.83
Day 10 – Pretransfusion	7.28 ± 1.10	7.21 ± 0.68
Day 10 – Post-transfusion	7.13 ± 0.93	6.99 ± 1.25
Day 21 – Pretransfusion	7.88 ± 0.93	7.85 ± 0.98
Day 21 – Post-transfusion	7.42 ± 1.59	7.77 ± 1.28

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

points. For the LR units, the $PGF_{2\alpha}$ concentrations in all of the subsequent samples, pre- and post-transfusion, were significantly ($P < .01$) lower than in the initial donation sample. There was no significant difference in $PGF_{2\alpha}$ concentration between the pre- and post-transfusion samples collected from the LR units on days 10 and 21. There was no difference in $PGF_{2\alpha}$ concentration among the samples collected from the non-LR units.

At the time of donation, the TXB_2 concentrations in LR units were significantly ($P < .0001$) higher than in the non-LR units. The TXB_2 concentrations in the LR units then decreased rapidly with storage, such that there was no difference in TXB_2 concentration between the LR and non-LR units for any of the remaining time points. The TXB_2 concentration in all LR samples, except for day 10 post-transfusion, was significantly ($P < .05$) decreased compared to the donation sample. There was no significant difference in TXB_2 concentration between the pre- and post-transfusion samples collected from the LR units on days 10 and 21. There was no difference in TXB_2 concentration among the samples collected from the non-LR units.

When compared to the initial donation sample, there was a significant ($P < .0001$) increase in 6-keto-$PGF_{1\alpha}$ concentration regardless of unit type for both LR and non-LR units on both days 10 and 21 for both the pre- and post-transfusion samples. The day 10 post-transfusion sample concentrations were significantly higher ($P = .0029$) than the day 21 pretransfusion samples, regardless of unit type. The 6-keto-$PGF_{1\alpha}$ concentrations for the LR units were significantly increased ($P = .0163$) compared to the non-LR unit concentrations, regardless of sample time.

Discussion

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

Based on our study, erythrocyte PS expression was not altered by leukoreduction, length of storage, or simulated transfusion. Similar findings have been reported in studies of humans, in which only a small percentage of PS-positive erythrocytes (3.5–4.5%) were detected after prolonged storage times of up to 7 weeks using similar techniques.[11,24–26] One possible explanation for why an increase in RBC PS expression was not detected is that the PS-positive portions of the erythrocyte membrane could have been released from the cell and

Table 2. The eicosanoid concentration (nM) (mean ± standard deviation) in units of canine pRBCs, with and without leukoreduction, at collection (donation) and after various lengths of storage (pretransfusion) and after a simulated transfusion (post-transfusion).

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

nM, nanomolar; pRBCs, packed red blood cells; Non-LR, nonleukoreduced; LR, leukoreduced; AA, arachidonic acid; PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TXB_2 thromboxane B_2; 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; LTB_4, leukotriene B_4.

Results labeled "*" illustrate significant ($P \leq 0.05$) differences from the respective donation sample.

Results labeled "**" illustrate significant ($P \leq 0.05$) differences between non-LR and LR samples at the same time point.

become microparticles in the unit. In stored units of human pRBCs, erythrocytes can undergo vesiculation to return to a PS-negative state by shedding PS-containing vesicles.[9,27] It is unknown when stored erythrocytes maximally express PS, but PS expression probably occurs continuously, followed by vesicle formation. In a previous study evaluating units of canine RBC concentrates, PS expression on the surface of microparticles increased during storage.[28] Therefore, if the PS-positive microparticles in units of canine pRBCs were released from erythrocytes, then PS expression on individual red blood cells would undergo minimal change during leukoreduction, storage, and transfusion.

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

In a previous study performed in dogs, storage of units of pRBCs caused a decrease in AA concentration, followed by a progressive increase in the concentration of eicosanoids in the units.[15] One possible explanation for these changes in eicosanoids during storage was a decline of cell health, particularly in leukocytes and platelets, leading to a decrease in cell viability and an increase in enzymatic conversion of AA to the various eicosanoids. In humans, prestorage removal of leukocytes and platelets by leukoreduction decreases the concentrations of PGE_2 and TXB_2 in stored units of pRBCs.[29] In contrast, in our study, the removal of leukocytes and platelets before storage did not significantly decrease eicosanoid concentrations. At most sample time points in our study, eicosanoid concentrations were similar between LR and non-LR units, suggesting that leukoreduction may have minimal impact on the accumulation of eicosanoids in units of canine pRBCs.

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

A possible explanation for the increase in TXB_2 concentration immediately after leukoreduction is that platelets are activated as they adhere to the fibers within the filter. In human blood products, based on platelet shape change and pseudopod formation, platelets appear to become activated as they pass through leukoreduction filters.[30] Once activated, platelets will increase expression of integrins needed for aggregation, change shape to cover a greater surface area, empty the contents of granules, and release AA to be converted to thromboxane A_2.[31] After synthesis, thromboxane A_2 has a very short half-life and is nonenzymatically degraded to the stable metabolite TXB_2.[32] Although platelets are the primary source of thromboxane A_2, several other cells are capable of synthesizing this eicosanoid, including leukocytes and endothelial cells.[32,33] Although the cyclooxygenase (COX)-2 enzyme has been identified in canine platelets,[34] COX-1 is the primary isoform in platelets, and probably is responsible for the increased synthesis of TXB_2 in units of pRBCs. It is unknown how increased concentrations of TXB_2 affect the patient, but the increased TXB_2 associated with leukoreduction and immediate transfusion has the potential to adversely impact the recipient.

In addition to TXB_2, $PGF_{2\alpha}$ also significantly increased immediately after leukoreduction. Prostaglandin $F_{2\alpha}$ is synthesized via 3 pathways from PGE_2, PGD_2, or prostaglandin H_2 (PGH_2) by PGE 9-ketoreductase, PGD 11-ketoreductase, or PGH 9-,11-endoperoxide reductase, respectively.[35] Collectively referred to as prostaglandin F synthases (PGFS), these enzymes are expressed in numerous organs including liver, lung, brain, kidneys, and uterus, but PGFS mRNA also has been identified in peripheral blood lymphocytes.[36] The mechanism of increased $PGF_{2\alpha}$ after leukoreduction in our study is unclear, but PGFS may have been released from lymphocytes activated during the filtration process. Some studies have described leukocyte activation with the use of leukocyte depletion filters,[37–39] but these findings were not consistently replicated in other leukoreduction studies.[40–43] Furthermore, leukocyte rupture is possible with prolonged filtration, resulting in free enzymes and other cellular components.[44] Similar to TXB_2, the increase in $PGF_{2\alpha}$ concentration after leukoreduction is particularly concerning because $PGF_{2\alpha}$ is associated with vasoconstriction and bronchoconstriction, which could impact the hemodynamic stability of transfusion recipients.[16]

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

Compared to our previous study,[15] despite some similar trends, overall the eicosanoid concentrations in our current study were significantly lower. One potential explanation for these differences is the quantity of cells in the unit. Unlike the previous study, which used greyhounds as donor dogs, our present study used hound dogs which, compared to greyhounds, have a lower percentage of circulating erythrocytes. The average hematocrit of the dogs in our current study was 49.5%, whereas the average hematocrit in our previous study was 68%. The lower percentage of cells in the units could have contributed to this decrease in eicosanoid synthesis.

Biosynthesis of eicosanoids depends on the availability of AA, which is derived from membrane phospholipids by several enzymes, including phospholipase A_2. Once released, AA can be metabolized by 3 main pathways: the COX pathway that produces prostanoids, the lipoxygenase (LOX) pathway that produces leukotrienes and lipoxins, and the P-450 epoxygenase pathway that produces epoxides.[16] The erythrocyte membrane is a rich source of AA, but lacks both the COX and LOX enzymes, and therefore cannot produce the majority of eicosanoids.[18] However, the AA-rich erythrocytes can contribute to eicosanoid synthesis by transcellular biosynthesis (ie, the cooperation of different cell types to produce eicosanoids). During transcellular biosynthesis, 1 cell type can synthesize an intermediate compound, through a primary oxidative enzyme, which is transferred to a neighboring cell to complete the final synthesis.

In our study, the only eicosanoid that increased in concentration during storage or simulated transfusion was 6-keto-$PGF_{1\alpha}$. This finding was similar to that of a previous study that identified an increase in 6-keto-$PGF_{1\alpha}$ concentration in units of canine pRBCs during storage and transfusion.[15] This prostaglandin is produced by nonenzymatic conversion from PGI_2, which is derived from the vascular epithelium or lymphocytes.[16] Interestingly, after removal of the leukocytes and platelets by leukoreduction, the predominate cell type expected to be remaining in the unit is the erythrocyte, which is not capable of synthesizing prostanoids. Therefore, we presume that, despite leukoreduction, components within units of pRBCs contributed to increased concentrations of 6-keto-$PGF_{1\alpha}$ by a process similar to transcellular biosynthesis. Specifically, AA could have been derived from the rich phospholipid bilayer of erythrocytes, and converted to prostacyclin by the COX-2 enzymes found in other cellular components in units of pRBCs.

Although it is unknown how the accumulation of eicosanoids in units of pRBCs will affect physiologic responses in the transfusion recipient, considering the functions of these molecules, the administration of blood products with increased concentrations of eicosanoids could have detrimental effects. For example, thromboxane promotes platelet aggregation, vasoconstriction, and bronchoconstriction, all of which may adversely affect hypercoagulable and hypertensive patients.[16] Thromboxane also can enhance the production of interleukin-8, a pro-inflammatory cytokine and potent neutrophil chemoattractant,[48,49] and therefore may promote transfusion-associated inflammatory responses. Additionally, transfusing blood products with high concentrations of prostacyclin, which inhibits platelet aggregation and promotes vasodilatation,[16] may further exacerbate complications associated with systemic hypotension in critically ill patients. Although our study did not detect significant accumulation of eicosanoids, additional studies under a range of different storage conditions are needed to determine whether eicosanoids can accumulate in storage to a concentration high enough to cause adverse effects in the recipient. To determine the effectiveness of leukoreduction, leukocyte and platelet counts were performed immediately before and after leukoreduction. The filter was effective at removing all leukocytes, but the filters were less effective at removing platelets before storage. Seven units had a post-filter platelet count of <20,000/μL, but 1 unit had 137,000/μL platelets post-filtration, a 60% reduction in platelet count compared to the prefiltration sample. This increased platelet count in the LR units could have altered the results of our study. If the eicosanoid and PS results from this unit were removed from our analyses, however, there was no major change in overall results, and the results from this unit therefore were included in our final analyses.

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

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

Footnotes

[a] Teruflex Optisol Triple Collection Blood Bag, Terumo Corporation, Tokyo, Japan
[b] Imuflex-WB-RP Blood Bag with integral whole blood leukocyte reduction filter, Terumo Corporation.
[c] Abbott Cell-Dyn® 3700, Abbott Laboratories, Abbott Park, IL
[d] Bovine Serum Albumin, Sigma-Aldrich, St. Louis, MO
[e] FITC Annexin V/Dead Cell Apoptosis Kit, Life Technologies, Grand Island, NY
[f] FACSCalibur, BD Biosciences, San Jose, CA
[g] CellQuest Pro software, BD Biosciences
[h] HyperSep Retain PEP 60 mg, 1 mL, Thermo Fisher Scientific, Waltham, MA
[i] Acquity UPLC BEH C18 column, Waters Corporation, Milford, MA
[j] TSQ Quantum Access Max, Thermo Fisher Scientific Inc
[k] Xcaliber software, ThermoFisher Scientific Inc, San Jose, CA
[l] SAS for Windows version 9.4, SAS Institute, Inc., Cary, NC

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Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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