Procoagulant phospholipid concentration in canine erythrocyte concentrates stored with or without prestorage leukoreduction

Stephanie A. Smith DVM, MS
Thandeka R. Ngwenyama DVM
Mauria O'Brien DVM
Jennifer M. Herring DVM
Rafaella Corsi DVM
Alyssa Galligan
Alison N. Beloshapka PhD
Ping Deng PhD
Kelly S. Swanson PhD
Maureen McMichael DVM

Received August 15, 2014.
Accepted September 9, 2014.

From the Department of Biochemistry, School of Molecular and Cellular Biology (Smith); the Department of Veterinary Clinical Sciences, College of Veterinary Medicine (Ngwenyama, O'Brien, Herring, Corsi, Galligan, McMichael); and the Department of Animal Sciences, College of Agricultural, Consumer and Environmental Sciences (Beloshapka, Deng, Swanson), University of Illinois, Urbana, IL 61801. Dr. Ngwenyama's present address is Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99164. Dr Herring's present address is Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37966. Dr. Corsi's present address is Department of Clinical Studies, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104.

Address correspondence to Dr. McMichael (mmcm@illinois.edu).

OBJECTIVE
To evaluate canine erythrocyte concentrates (ECs) for the presence of procoagulant phospholipid (PPL), determine whether PPL concentration changes during the course of storage of ECs, and ascertain whether prestorage leukoreduction (removal of leukocytes via gravity filtration) reduces the development of PPL.

SAMPLE
10 whole blood units (420 g each) collected from 10 random-source, clinically normal dogs (1 U/dog).

PROCEDURES
The dogs were randomized to 1 of 2 groups. Of the 10 whole blood units collected, 5 were processed through a standard method, and 5 underwent leukoreduction. Whole blood units were processed to generate ECs, from which aliquots were aseptically collected from each unit weekly for 5 weeks. Supernatants from the concentrates were evaluated for procoagulant activity, which was converted to PPL concentration, by use of an automated assay and by measurement of real-time thrombin generation.

RESULTS
Supernatants from stored canine ECs contained procoagulant activity as measured by both assays. In general, the PPL concentration gradually increased during the storage period, but leukoreduction reduced the development of increased procoagulant activity over time.

CONCLUSIONS AND CLINICAL RELEVANCE
The presence of PPL in canine ECs may be associated with procoagulant and proinflammatory effects in vivo, which could have adverse consequences for dogs treated with ECs. (*Am J Vet Res* 2015;76:35–41)

Delivery of oxygen-carrying capacity via transfusion of ECs (also known as packed RBCs) is a valuable support mechanism in the management of critically ill humans and other animals. Despite the well-documented benefits of transfusion, administration of ECs carries the potential for a variety of adverse events, some of which are a consequence of the duration of storage of the cells.[1]

During storage, erythrocytes undergo various changes that are generally referred to as RBC storage lesions.[2] Storage-related effects may impact cell membranes, leading to the generation of microparticles.[3] Microparticles are small (generally < 1 µm in diameter), cytosol-containing vesicles surrounded by a membrane bilayer. Microparticles may develop under normal physiologic conditions from platelets and a variety of cells, including erythrocytes, endothelial cells, and leukocytes. Erythrocyte-derived microparticles are formed only in very low numbers in vivo under normal physiologic conditions,[4] but marked generation of microparticles from all hematologic cell types during storage of human ECs has been reported.[3,5-7]

Microparticles are involved in several processes,[5] including inflammation,[8] angiogenesis,[9] hemostasis,[8] thrombosis,[10] infection,[11] and endothelial function.[12] The presence of PPLs such as phosphatidylserine on the surface of microparticles allows binding of coagulation factors[13] and is necessary for normal thrombin production.[8] Microparticles derived from stored human ECs are able to support thrombin generation in vitro.[14]

Leukoreduction (removal of leukocytes by a specially charged filter) has the potential to decrease microparticle formation in stored ECs through elimination of many of the cell types from which they are formed during the storage period, as well as through reduction of proinflammatory mediators that are generated by leukocytes and platelets. The objectives of the study reported here were to evaluate canine ECs

ABBREVIATIONS

EC	Erythrocyte concentrate
PPL	Procoagulant phospholipid

for the presence of PPL, determine whether PPL concentration changes during the course of storage of ECs, and ascertain whether prestorage leukoreduction reduces the development of PPL.

Materials and Methods

COLLECTION OF SUPERNATANT SAMPLES

Whole blood units were collected from 10 random-source healthy research dogs (1 U/dog) and processed exactly as previously described.[15] The study was approved by the University of Illinois Institutional Animal Care and Use Committee. Briefly, 420 g of whole blood was collected from each donor dog into citrate phosphate dextrose adenine. Five of the units were collected into standard triple blood bags[a] (nonleukoreduced units), and 5 of the units were collected into triple blood bags that contained an in-line leukoreduction filter system[b] (leukoreduced units) to remove leukocytes and platelets. Leukoreduction was performed at room temperature (approx 20°C) via gravity filtration through the in-line filter on units randomized to the leukoreduction group. In leukoreduced units, cell and platelet depletion was > 99% as confirmed by automated cell counts and by manual counts performed by a board-certified pathologist. Whole blood units (both leukoreduced and nonleukoreduced) were then centrifuged at 5,010 × *g* for 15 minutes at 10°C for removal of plasma and concentration of erythrocytes. Cell preservative (100 mL) was added to each leukoreduced[c] unit with a sterile technique and was added to each nonleukoreduced[d] unit from within the collection system. The EC units were stored upright at 4°C for the duration of the study (35 days), with repeated gentle inversion performed every 2 days and immediately before sterile collection of aliquots.

Sterile ports were placed in each unit to allow for collection of samples at baseline (day 0 [day of phlebotomy]) and once weekly during a 5-week storage period. Samples were aseptically withdrawn from each unit via attachment of a sterile syringe to a sampling port. Port placement and sample collection were always performed in a laminar airflow workbench with aseptic technique and sterile supplies. On day 35, following collection of samples, a 1-mL aliquot was obtained from each unit and submitted for aerobic bacterial culture. Samples were centrifuged at 1,850 × *g* for 20 minutes at 4°C without braking. The upper two-thirds portion of each sample (defined as supernatant) was collected and aliquoted. Each supernatant aliquot was either evaluated immediately (designated as fresh) or stored at –80°C (designated as frozen) for later (approx 4 months) batch analysis.

PPL ASSAY

The procoagulant activity of the phospholipids within the EC supernatant samples was evaluated with a phospholipid assay[e] in duplicate on a coagulometer[f] at 37°C according to the manufacturer's instructions, with minor modifications as follows: 5-fold dilutions of EC supernatant aliquots were generated with buffer,[g] and clot times were converted to PPL concentration against a standard curve (0.2 to 12 µg/mL) determined by use of synthetic liposomes (prepared by sonication) that contained 20% phosphatidylserine and 80% phosphatidylcholine.[h] The EC supernatant samples were evaluated both within 1 hour after preparation and following storage of approximately 4 months' duration at –80°C.

CALIBRATED AUTOMATED THROMBOGRAPHY

The ability of the phospholipid within EC supernatant samples to support thrombin generation was evaluated via calibrated automated thrombography[i] by the Hemker method with the manufacturer-supplied reagents.[j-l] Calibrated automated thrombography determines the concentration of thrombin in clotting plasma by monitoring the cleavage of a fluorogenic substrate and comparing the rate of the cleavage with

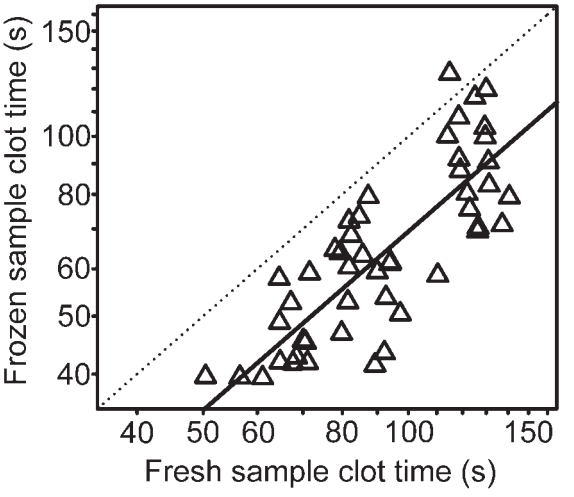

Figure 1—Plot of clot times determined by use of an automated assay for diluted fresh EC supernatant samples and frozen EC supernatant samples derived from whole blood units (420 g of whole blood each) collected from 10 random-source healthy research dogs (1 U/dog). Five of the units were collected into standard triple blood bags (nonleukoreduced units), and 5 of the units underwent leukoreduction be means of an in-line filter system to remove leukocytes and platelets. Whole blood units (both leukoreduced and nonleukoreduced) were then centrifuged for removal of plasma and concentration of erythrocytes. The EC units were stored upright at 4°C for the duration of the study (35 days) with repeated gentle inversion performed every 2 days and immediately before sterile collection of samples for analysis. Samples were removed for analysis at baseline (day 0 [day of phlebotomy]) and once weekly during a 5-week storage period. Each sample underwent centrifugation, and the upper two-thirds portion (defined as supernatant) was collected and aliquoted on days 0, 7, 14, 21, 28, and 35. Each aliquot was either evaluated immediately (designated as fresh) or stored at –80°C for later analysis (designated as frozen and analyzed following thawing on a single day as a batch analysis). In this plot, data for 49 samples are represented, and the dotted line represents equivalence. The solid line indicates linear regression. Data for the fresh and frozen EC supernatant samples were significantly ($P < 0.001$) positively ($r = 0.815$) correlated, but there was marked bias toward longer clot times for the fresh samples.

a constant known thrombin activity in a parallel, nonclotting sample. This assay evaluates the time course of thrombin generation and inhibition in real time, reflecting the ongoing generation of thrombin that occurs after formation of the fibrin clot. Ten-fold dilutions of EC supernatant samples (source of phospholipid) were generated with phospholipid-depleted plasma (source of zymogen coagulation factors), which was prepared by centrifugation of healthy human pooled plasma[m] at 20,000 × *g* for 30 minutes.

STATISTICAL ANALYSIS

Normality was established with the Kolmogorov-Smirnov test. Correlation of clot times between fresh and frozen samples was determined with the Pearson product moment correlation. Data points were compared by 2-way repeated-measures ANOVA with evaluation for an effect of both group (nonleukoreduced units vs leukoreduced units) and storage time (0, 7, 14, 21, 28, or 35 days). A value of $P < 0.05$ was considered significant. Comparisons were performed with a statistical software program.[n]

Results

PPL ASSAY

Clot times obtained for diluted fresh EC supernatant samples (performed on multiple different days) and those obtained for the frozen EC supernatant samples (performed on a single day as a batch analysis) were assessed and compared **(Figure 1)**. Clot times for the fresh and frozen EC supernatant samples were significantly ($P < 0.001$) and positively ($r = 0.815$) correlated, although there was a bias toward longer clot times (less procoagulant activity) in fresh samples.

Procoagulant phospholipid concentrations in frozen EC supernatants as a function of the duration of EC storage were evaluated **(Figure 2)**. Although the raw measurements of PPL concentration were different depending on whether the supernatant was tested before or after freezing, the comparison between fresh or frozen leukoreduced and nonleukoreduced units was similar, as was the comparison of the effect of the duration of storage of the EC (data not shown). There were significant effects of day of storage ($P < 0.001$) and group (leukoreduced units vs nonleukoreduced units; $P < 0.001$) as determined by 2-way repeated-

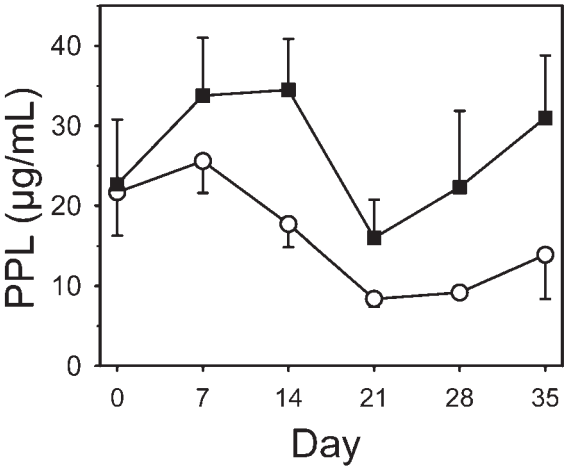

Figure 2—Concentrations of PPL in the leukoreduced (circles) and nonleukoreduced (squares) canine EC supernatant samples described in Figure 1 on days 0, 7, 14, 21, 28, and 35 of storage. Phospholipid-containing supernatant samples were collected from ECs following centrifugation, underwent 5-fold dilution, and were evaluated after freezing by use of an automated assay in a single-batch assay. Coagulation times were converted to phospholipid concentration by means of a standard curve generated from locally prepared phospholipid vesicles. Data points (n = samples from 5 dogs for each group) represent the mean, and error bars represent the SD. When analyzed by 2-way repeated-measures ANOVA, there were significant ($P < 0.001$) effects of both day of storage and group (leukoreduced vs nonleukoreduced). *See* Figure 1 for remainder of key.

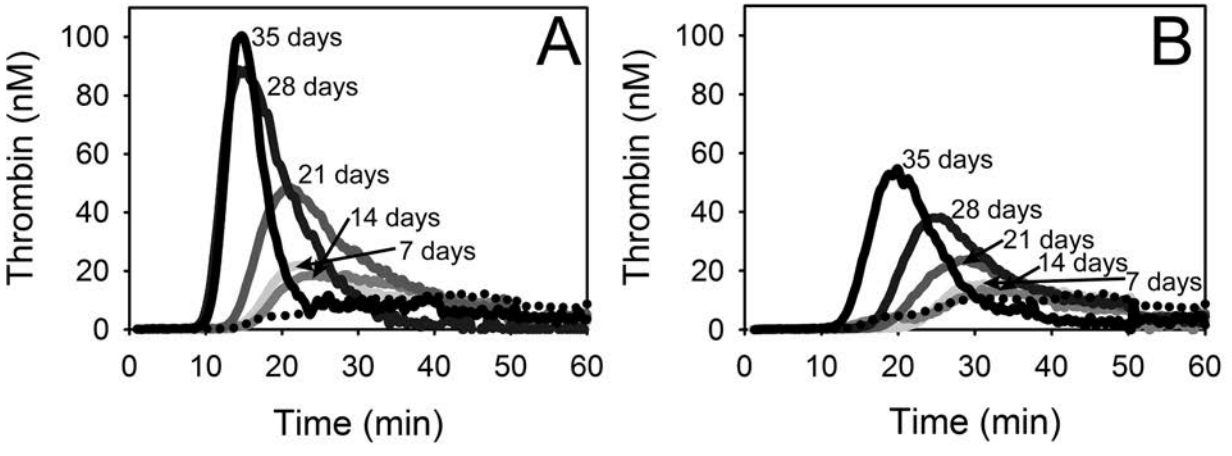

Figure 3—Thrombin generation curves for diluted nonleukoreduced (A) and leukoreduced (B) supernatant samples from canine ECs described in Figure 1 on days 0, 7, 14, 21, 28, and 35 of storage. Samples were added to pooled phospholipid-depleted plasma from healthy humans, and thrombin generation was initiated via addition of a low concentration of tissue factor. The concentration of thrombin is plotted against the time after initiation of thrombin generation. The curves represent the mean (n = samples from 5 dogs) thrombin concentration at each time point. The dotted line represents thrombin generation in the plasma in the absence of added phospholipid. ***See*** Figure 1 for remainder of key.

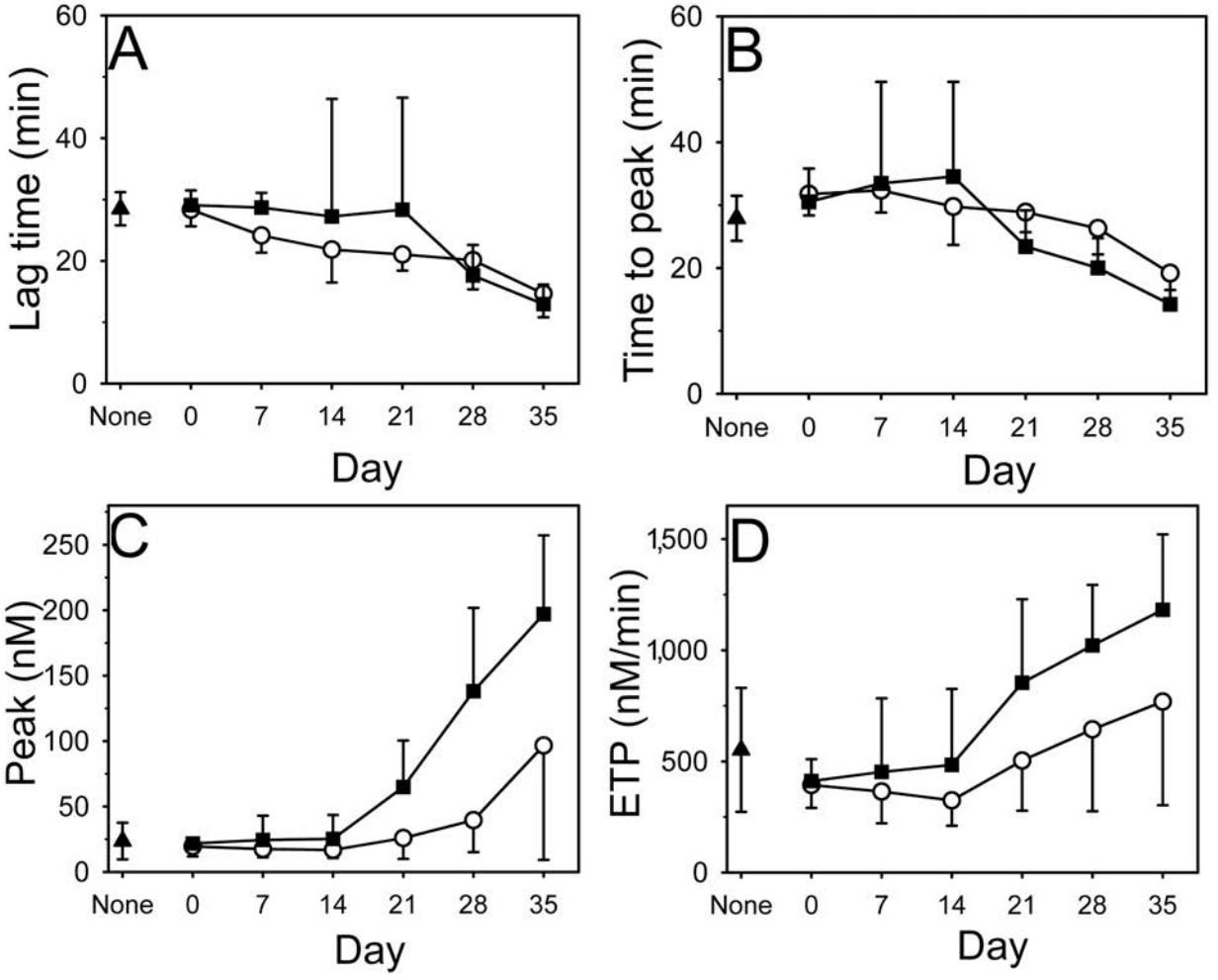

Figure 4—Variables determined by calibrated automated thrombography for diluted nonleukoreduced (squares) and leukoreduced (circles) supernatant samples from canine ECs described in Figure 1 on days 0, 7, 14, 21, 28, and 35 of storage and in pooled phospholipid-depleted plasma from healthy humans without any added supernatant sample (triangle). Samples were added to pooled phospholipid-depleted healthy human plasma, and thrombin generation was initiated by addition of tissue factor. Variables of interest were lag time to initiation of thrombin generation (A), time to peak thrombin concentration (B), peak thrombin concentration (C), and endogenous thrombin potential (ETP, which is representative of the integrated area under the curve [D]). For each group, data points represent the mean (n = samples from 5 dogs), and error bars represent the SD. When analyzed by 2-way repeated-measures ANOVA, there were significant ($P < 0.001$) effects of both day of storage and group (leukoreduced vs nonleukoreduced). ***See*** Figure 1 for remainder of key.

measures ANOVA. The PPL concentrations in leukoreduced and nonleukoreduced units were similar on storage days 0 and 7, but were significantly higher in nonleukoreduced units on days 14, 21, 28, and 35. The PPL concentration decreased over the initial 21 days of storage in both leukoreduced and nonleukoreduced units; however, there was an approximately 2-fold increase in the PPL concentration in nonleukoreduced units during the last 14 days of storage.

CALIBRATED AUTOMATED THROMBOGRAPHY

From the thrombin generation curves **(Figure 3)**, variables of interest were derived **(Figure 4)**. Thrombin generation in the phospholipid-depleted human plasma was minimal in the absence of supernatant-supplied PPLs. The ability of phospholipid in EC supernatant samples to support thrombin generation increased significantly over the period that the ECs were stored, as indicated by an increase in peak thrombin concentration and endogenous thrombin potential. The EC storage-related change occurred regardless of whether the whole blood unit had undergone leukoreduction, but nonleukoreduced units were significantly better at supporting thrombin generation than were leukoreduced units.

Discussion

Microparticles that accumulate in ECs during a storage period are considered to be part of the storage lesion.[16] Because multiple clinical studies[17–20] have suggested that the interval since preparation of transfused ECs may be linked to recipient outcome in humans, it

has been proposed that microparticles in stored blood units may contribute to the potential adverse effects of transfusion.[3] The production of microparticles in ECs as a function of storage time has been documented for human,[6,14,21,22] mouse,[23] and canine blood.[15] These microparticles appear to be primarily derived from erythrocytes,[3] but leukocyte- and platelet-derived microparticles may also be present.

Results of previous research with human ECs have suggested that a component of the supernatant from ECs is able to support thrombin generation,[22,24] and recent evidence suggests that microparticles are the major contributing element.[14,25] It is apparent from the data obtained in the present study that the supernatant of stored canine ECs similarly contains considerable procoagulant activity and that prolonged storage increases the procoagulant activity. Erythrocyte-derived microparticles produced in vitro are reported to have procoagulant properties ex vivo and reduce bleeding in rabbits and rats.[26] Consequently, erythrocyte-derived microparticles have been proposed as a potential prohemostatic agent for therapeutic use in animals with bleeding disorders.[26] It is possible that, in the future, blood may be processed to contain a high concentration of microparticles to be used for treatment of anemia from certain disease states (eg, hemophilia).

Results of the present study indicated that less procoagulant activity developed over time in ECs that underwent prestorage leukoreduction than in nonleukoreduced ECs. This finding is consistent with the finding of our previous study[15] that leukoreduction reduced the development of phosphatidylserine-expressing microparticles in canine ECs, and with similar findings for human ECs.[27] Interestingly, removal of leukocytes and platelets by means of leukoreduction appears to decrease the production of not just leukocyte- and platelet-derived microparticles, but of erythrocyte-derived microparticles as well.[3]

Both of the assays we used in the present study to measure the ability of EC supernatant to provide procoagulant activity are designed to maximize sensitivity specifically for detection of PPLs (as opposed to other procoagulant substances). This is in part because plasma depleted of lipid is used to provide the zymogen coagulation factors needed for generation of thrombin. As a consequence, the small amount of phospholipid provided by the diluted supernatant sample is the limiting factor in the generation of thrombin. In the phospholipid assay, wherein thrombin is generated with the addition of factor Xa, the time to clot formation is primarily a function of the ability of the prothrombinase complex to assemble on the provided membrane surface.[28,29] In contrast, we used a low concentration of tissue factor to initiate the reaction in the thrombin generation assay; thus, in this assay, the membrane would additionally need to provide a surface for assembly of the intrinsic tenase complex.

The procoagulant activity of the supernatant samples in the present study was likely primarily a function of the expression of phosphatidylserine on the outer leaflet of the membrane bilayer. Phosphatidylserine expression is required for binding of membrane-dependent coagulation enzyme complexes.[30] Our previous work revealed the presence of this anionic phospholipid on microparticles in supernatant from canine ECs by flow cytometry to detect the binding of annexin-V to phosphatidylserine.[15] Phosphatidylserine expression on microparticles derived from human ECs has also been documented,[21,22,31,32] although 1 study[31] revealed a decrease in phosphatidylserine expression over storage time, despite an increase in microparticle concentration.

It is possible that some of the procoagulant activity in the EC supernatant samples evaluated in the present study was associated with tissue factor–bearing microparticles. The coagulation assay is designed to minimize this effect by addition of a downstream enzymatic trigger (factor Xa), but because tissue factor is a potent activator, it is possible that endogenously generated factor Xa could have contributed somewhat to the shortening of coagulation time. Similarly, the design of the thrombin generation assay would not have prevented extrinsic tenase activity associated with tissue factor within the diluted samples. It is unlikely though that tissue factor was a major contributor to the procoagulant nature of these supernatants because erythrocyte-derived microparticles do not contain tissue factor.[14] Furthermore, results of previous studies[14,22] with human EC-derived microparticles suggest that the procoagulant activity is independent of the presence of tissue factor.

An additional limitation of the present study was related to the methods used to prepare the EC supernatant samples for analysis. On the basis of previous reports, we elected to apply a centrifugation force of 1,850 × *g*.[6] The centrifugal force applied clearly impacts the quantity and size distribution of the microparticles that remain in the supernatant.[6] If we had used a different force, we would expect that the raw quantity of PPL recovered would have been different; however, because an identical processing protocol was used for all samples, we believe that the comparisons with respect to group and duration of storage were not impacted. Similarly, the comparison of PPL concentrations measured with the coagulation assay in freshly analyzed samples with those analyzed in samples after freezing indicated that freezing did have an impact on the total amount of PPL measured. However, regardless of whether the samples were fresh or frozen, the effects of the group (nonleukoreduced or leukoreduced units) and duration of storage were identical. Owing to the nature of the assay, frozen samples were only evaluated by calibrated automated thrombography as a batch analysis.

In addition to the described procoagulant activity associated with ECs, it appears that the transfusion of microparticles derived from stored human and murine ECs may also cause a variety of proinflammatory effects. In an in vitro study,[32] human EC-derived

microparticles fixed the first protein of the complement cascade (C1q), leading to activation of the classical pathway. These microparticles also inhibited macrophage activation by agonists such as endotoxin[32] and were able to prime neutrophils in vitro.[33] Microparticles from stored human ECs have an altered ability to bind chemokines and have an increased propensity to release these chemokines when they interact with platelets.[33]

Studies in mice have similarly revealed roles for erythrocyte-derived microparticles in inflammatory responses in vivo. Injection of microparticles from murine ECs into healthy laboratory mice results in neutrophil priming, and resuscitation with these microparticles in mice with hemorrhage causes a marked increase in pulmonary neutrophil accumulation[23,34] and histologic changes in the lungs.[23] Endotoxin-induced peripheral blood leukopenia is aggravated by injection of EC-derived microparticles, and plasma proinflammatory cytokine concentrations are markedly increased after injection. These effects appear to be thrombin and phosphatidylserine dependent.[34] Results of previous work by our group[35] and others[36] in dogs indicate that transfusion of stored ECs is also clearly associated with an inflammatory response.

The results of the present study have indicated that supernatants from stored canine ECs contain procoagulant activity. The procoagulant activity is likely to be associated with procoagulant and proinflammatory effects in vivo, which could have adverse consequences for dogs treated with such ECs. In the present study, the procoagulant activity appeared to gradually increase during storage, but prestorage leukoreduction reduced the development of increased procoagulant activity over time. Additional investigations are indicated to further evaluate the potential impact of prestorage leukoreduction on outcome in canine EC recipients.

Acknowledgments

The Pall Corp donated the leukoreduction filter bags, Jerry Fina of Charter Medical donated the sampling ports, and Paul Riley of Diagnostica Stago donated the STA Procoag-PPL kits.

Presented in part at the International Veterinary Emergency and Critical Care Symposium, San Antonio, Tex, September 2012.

The authors thank Jessica Garrett for technical assistance.

Footnotes

a. Terumo Transfusion Products, Somerset, NJ.
b. Leukotrap WB System, Pall Corp, East Hills, NY.
c. Adsol, Baxter Healthcare, Deerfield, Ill.
d. Optisol, Terumo Transfusion Products, Somerset, NJ.
e. STA Procoag-PPL, Diagnostica Stago, Asnieres, France.
f. STart4 coagulometer, Diagnostica Stago, Asnieres, France.
g. Owren-Koller buffer, Diagnostica Stago, Asnieres, France.
h. Avanti Polar Lipids, Alabaster, Ala.
i. Thrombinoscope BV, Maasstraat, The Netherlands.
j. PPP-Low Reagent, Thrombinoscope BV, Maasstraat, The Netherlands.
k. FluCa, Thrombinoscope BV, Maasstraat, The Netherlands.
l. Thrombin Calibrator, Thrombinoscope BV, Maasstraat, The Netherlands.
m. Citrated human pooled normal plasma, George King Biomedical, Overland Park, Kan.
n. Sigma Stat, version 2.03, SPSS Inc, Chicago, Ill.

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