Assessment of a dimethyl sulfoxide–stabilized frozen canine platelet concentrate

Julien Guillaumin, Doct. Vet.; Karl E. Jandrey, DVM; Jeffrey W. Norris, PhD; Fern Tablin, VMD, PhD

Objective—To assess platelet count, mean platelet volume (MPV), metabolic characteristics, and platelet function in a dimethyl sulfoxide (DMSO)–stabilized canine frozen platelet concentrate (PC).

Sample Population—11 units of a commercial frozen PC in 6% DMSO and fresh platelet-rich plasma from 6 healthy control dogs.

Procedures—PCs were thawed, and the following data were collected: thaw time, platelet count, MPV, pH, Pco₂, and Po₂ and HCO₃⁻; glucose, and lactate content. Phosphatidylserine translocation was determined by use of flow cytometry. Fresh platelet-rich plasma from healthy dogs served as a source of control platelets for flow cytometric analysis.

Results—At thaw, the platelet count in the frozen PC ranged from 243,000 to 742,000 platelets/µL. Median platelet count of paired samples was 680,000 platelets/µL and decreased significantly to 609,000 platelets/µL at 2 hours after thaw. Median MPV at thaw was 11.15 femtoliters and was stable after 2 hours. Compared with fresh platelets, frozen PC had increased amounts of phosphatidylserine in the outer leaflet of the platelet membrane in the resting (ie, not treated with thrombin) state (19% vs 99%, respectively) and alterations in cellular morphology, all of which were consistent with platelet activation.

Conclusions and Clinical Relevance—Results of this in vitro study indicated that there was a decrease in platelet quantity and function as well as an increase in platelet activation during the freeze-and-thaw process in DMSO-stabilized canine frozen PC. In vivo effects on PC remain to be determined. (Am J Vet Res 2008;69:1580–1586)

Platelets are anucleate, discoid-shaped, cytoplasmic fragments that play an essential role in primary hemostasis, the initiation of a platelet plug, and the preservation of vascular integrity. Alterations in platelet number as well as platelet function have profound implications for the maintenance of hemostasis and vascular health. Thrombocytopenia, a common finding in critically ill dogs, is often associated with spontaneous bleeding in dogs and in humans. Moreover, progressive development of thrombocytopenia has been associated with a poorer outcome in human intensive care unit patients.

In human medicine, platelet-rich products are available for the treatment of patients with thrombocytopenia. The most commonly used product is a fresh PC stored at room temperature (20° to 24°C) with continuous, gentle agitation for up to 5 days. Fresh PC can be obtained from whole blood by serial centrifugation or by platelet apheresis. The minimum acceptable platelet count in a PC is 3.0 × 10¹¹ platelets/300-µL bag, as defined by the American Association of Blood Banks.

Cryopreservation, most commonly by use of DMSO as a cryopreservative, has also been used for the storage of human PCs. These DMSO-stabilized frozen PCs have a longer shelf life and are easier to ship, compared with stored fresh PC. Platelets in 6% DMSO have been stored at –80°C for up to 2 years. However, DMSO cryopreservation results in a substantial loss of platelets, altered platelet morphology, decreased response to agonists as evaluated by aggregation, and reduced clinical efficacy, compared with fresh platelets.

In veterinary medicine, a frozen PC is commercially available for dogs, which is prepared from a single donor by use of apheresis technology and cryopreserved with 6% DMSO. No published information exists regarding platelet function for this specific product. Limited information exists in the peer-reviewed veterinary literature regarding canine DMSO-stabilized frozen PC. These studies did not assess platelet function but rather evaluated platelet number and morphology. From this information, it can be concluded that cryopreservation of canine platelets with DMSO approaches that of human platelets. The present study is the first to assess platelet function in a DMSO-stabilized canine frozen PC.

A B B R E V I A T I O N S

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FTR</td>
<td>Freeze-thaw recovery</td>
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<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>MPV</td>
<td>Mean platelet volume</td>
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<td>PC</td>
<td>Platelet concentrate</td>
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<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
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<td>T0</td>
<td>Time zero</td>
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not analyze this commercial, or an equivalent, PC. Furthermore, differences in methods that make direct comparison difficult include blood collection (platelet apheresis vs serial centrifugation), storage temperature (–80°C vs –20°C), thawing process (warm vs room temperature), or presence versus absence of a washing step (to remove the cryopreservative prior to administration). The purpose of the study reported here was to assess hematologic and metabolic characteristics as well as platelet function in a commercially available canine frozen PC in 6% DMSO. We hypothesized that the PC would have adequate platelet numbers per bag (ie, 1.0 X 10^11 platelets/100-mL bag or 1.000 X 10^5 platelets/µL) and that the platelet function would be decreased, compared with that of fresh platelets.

Materials and Methods

Control dogs and platelet preparation—Blood was collected from 6 dogs owned by staff veterinarians or veterinary students from the Veterinary Medical Teaching Hospital at the University of California, Davis. Consent was obtained from owners prior to study enrollment; dogs and blood were handled according to institutionally approved protocols. Dogs (5 castrated males, 1 spayed female; median age, 5.5 years [range, 3 to 13 years]) were considered healthy on the basis of history, physical examination findings, and results of a CBC. None of the dogs had received drugs during the 10 days preceding blood collection.

Fresh whole blood was obtained by venipuncture and immediately transferred into a commercial specimen tube containing acid-citrate-dextrose to achieve a 1:7 acid-citrate-dextrose-to-blood ratio. Platelet-rich plasma was as well as platelet-rich plasma with 6% DMSO was prepared for subsequent studies. Platelet-rich plasma was prepared by centrifugation of whole blood at 2,000 X g for 3 minutes at 23°C within 30 minutes of blood collection. Platelet-poor plasma (ie, < 10,000 platelets/µL) was prepared by centrifugation of the platelet-rich plasma at 20,000 X g for 10 minutes. Dimethyl sulfoxide was added to the previously prepared platelet-rich plasma to achieve a final 6% concentration, gently mixed, and allowed to rest for ≥10 minutes at 37°C before analysis.

Platelet concentrate—Eleven units of frozen canine PC were purchased, and each PC was placed in a –20°C freezer immediately upon receipt according to the instructions of the manufacturer. All analyses were performed within 10 days of receipt of the product. The PC was thawed at room temperature, visually inspected, and gently swirled every 5 minutes during the thaw process. The time at which the PC was entirely fluid was defined as the thaw time. The PC was allowed to rest 15 minutes before starting analysis (T0), and analysis was completed within 4 hours of thawing, without washing the product.

Hematologic and metabolic analysis—At T0, pH, Pco2, and Po2 and HCO3−, glucose, and lactate content were measured with an intensive care unit analyzer. Thaw time was also recorded.

For hematologic analysis, platelet count and MPV were measured at T0 and T0 + 2 hours by use of an automated cell counter. The cell counter used a pulsatile change in the electrical field for cell count and sorted cells on the basis of size. Pulses that represent cells from 2 to 20 µL were classified as platelets. Platelet pulses were sorted by size into 64 channels to produce a platelet histogram, and the MPV was derived from the platelet histogram. Accuracy of results was maximized by triplicate counting and internal voting criteria.

Freeze-thaw recovery was calculated at T0 and T0 + 2 hours; it was defined as the concentration of platelets after thaw divided by the concentration of platelets stated by the manufacturer to be present in the bag prior to freezing (ie, 1 X 10^9/µL) multiplied by 100.

Annexin V labeling of surface phosphatidylserine and flow cytometry—Translocation of phosphatidylserine from the inner membrane leaflet of platelets to the outer membrane leaflet is an early indicator of platelet activation that can be detected by use of FITC-labeled annexin V, which specifically binds to phosphatidylserine. Five frozen PCs were used for the flow cytometry analysis. Fresh platelet-rich plasma and fresh platelet-rich plasma with 6% DMSO were used as a source of control platelets. Platelets in each product were diluted in Tyrode-HEPES buffer (12mM NaHCO3, 138mM NaCl, 2.9mM KCl, 10mM HEPES, 5mM dextrose; pH, 7.2). Calcium chloride from a 10mM stock in Tyrode-HEPES was added in 5 equal aliquots at 5-minute intervals to achieve a final concentration of 1mM CaCl2 and 3 X 10^7 cells/mL. The FITC-labeled annexin V was used to detect phosphatidylserine translocation to the outer membranes of platelets from control dogs and PC.

Fresh platelet-rich plasma, fresh platelet-rich plasma with 6% DMSO, and frozen PCs were analyzed at rest and after activation. Washed platelets were activated with thrombin (1 U/mL) for 15 minutes at 37°C.

### Table 1—Analyses completed on each PC.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Time</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tr>
<td>Platelet concentrate No.</td>
<td></td>
<td>T0</td>
<td>MDP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>Hematologic</td>
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<td>T0</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Metabolic</td>
<td></td>
<td>T0 + 2 h</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Flow cytometry</td>
<td></td>
<td>T0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

Hematologic analysis included platelet count, MPV, and FTR. Metabolic data included thaw time, pH, Pco2, and Po2 and HCO3−, glucose, and lactate content. T0 = Time at thaw, defined by the time to PC fluidity, plus 15 min. MDP = Missing data point. + = Assessed. – = Not assessed.
Platelets (5 \times 10^5) were removed from the reaction, diluted to 100 \muL with Tyrode-HEPES plus 1mM CaCl_2, and labeled with FITC-labeled annexin V according to the recommendation of the manufacturer. Flow cytometric analysis was performed after a 10-minute incubation at 37°C.

Forward and side scatter voltages were set to detect machine noise, which was removed during subsequent analyses. In experiments with FITC-labeled annexin V, the fluorescence channel 1 detector was set such that 95% of the autofluorescence of the control platelets was binned in the first logarithm of the detector response.

Statistical analysis—Nonparametric measures of comparison were used because platelet count, MPV, and FTR were not normally distributed. Differences between groups were assessed by use of a Mann-Whitney U test. Data were reported as median and range values (n = number of samples). Flow cytometry data were normally distributed; therefore, a Student t test was used for data analysis. A value of P < 0.05 was considered to be significant.

Results

Hematologic and metabolic analyses—Analyses were completed on 11 PCs (Table 1). Visual and microscopic examination of the PC at thaw revealed swirling platelets, platelet aggregates, and air bubbles. The aggregates were of different sizes, ranging from 2 mm to 2 cm in diameter. Over time (up to 6 hours), the swirling decreased, and aggregates decreased in number but increased in size (up to 5 to 6 cm in diameter). Median thaw time was 77.5 minutes (range, 70 to 100 minutes; n = 6).

Platelet count at T0 ranged from 243,000 to 742,000 platelets/\muL (median, 590,500 platelets/\muL; n = 10) with an FTR of 59%. Using the 5 PCs for which we had paired analyses, median platelet counts decreased from 680,000 platelets/\muL at T0 (range, 577,000 to 742,000 platelets/\muL; n = 5) to 509,000 platelets/\muL at T0 + 2 hours (range, 496,000 to 568,000 platelets/\muL; 5; P = 0.009). The FTR was 68% at the time recommended to begin a transfusion and decreased to 51% (P = 0.009) after 2 hours, the time recommended by the manufacturer to have concluded the transfusion.

Median MPV of the fresh platelets was 6.75 fL (range, 5.6 to 8.5 fL; n = 6). Median MPV of the PC was 11.15 fL at T0 (range, 9.6 to 13.8 fL; n = 10), significantly (P = 0.001) greater than that of fresh platelets. For the paired sample, median MPV was similar at T0 and T0 + 2 hours, 11.1 fL (range, 9.6 to 13.8 fL; n = 5) and 11.1 fL (range, 9.7 to 17.4 fL; 5), respectively (P = 0.83; Tables 2 and 3).

Platelets in the resting state obtained from frozen-thawed PCs were 99 ± 0.92% positive for phosphatidylserine on their surfaces and had a mean MFI of 1,303 ± 560 RFU (Figure 1). After thrombin activation of these platelets, 98 ± 0.82% were positive for phosphatidylserine and had a mean MFI of 1,400 ± 280 RFU. Therefore, detection of phosphatidylserine on platelets in the resting state obtained from frozen-thawed PC did not change significantly after stimulation with thrombin (P = 0.107 and P = 0.730 for percentage and RFU values, respectively).

The progressive activation of platelets in response to the DMSO treatment and cryopreservation was also apparent in the physical characteristics measured by use of flow cytometry, forward scatter and side scatter. After stimulation with thrombin, a typical population of untreated (ie, no treatment with DMSO) fresh platelets in the resting state shifted to lower forward and side scatter values (Figure 2), which corresponded to the change in shape that platelets undergo after activation. Activation of non-DMSO incubated fresh platelets with thrombin was also accompanied by a significant (P < 0.001) increase in the mean number of microparticles from 910 ± 140 to 6,570 ± 1,040. Incubation of fresh, resting platelets with 6% DMSO resulted in a split platelet distribution in which 32 ± 11% of the platelets had lower forward and side scatter values, characteristic of

Flow cytometry—In the absence of cryopreservative (ie, DMSO), the percentage of fresh platelets with detectable phosphatidylserine increased from 19 ± 2.5% in the resting state to 67 ± 7.7% after stimulation with thrombin (Figure 1). This increase in the percentage of fresh platelets with detectable phosphatidylserine was accompanied by an increase in the amount of phosphatidylserine on their surface, which was reflected by an increase in the MFI from 280 ± 52 RFU to 1,100 ± 220 RFU.

In contrast, 64 ± 14% of fresh platelets in 6% DMSO in the resting state had phosphatidylserine detected on their surface with a mean MFI of 1,200 ± 460 RFU. These results were not significantly different from the treatment of resting platelets with thrombin (P = 0.761 and P = 0.751 for percentage and RFU values, respectively). The fresh platelets in 6% DMSO responded to thrombin, although this response was not significant. After stimulation with thrombin, the percentage of fresh platelets in 6% DMSO that were positive for annexin V was 84 ± 3.2% and had a mean MFI of 1,900 ± 310 RFU (P = 0.174 and P = 0.119 for percentage and RFU values, respectively, compared with fresh platelets in 6% DMSO in the resting state).

Table 2—Median (range) hematologic values of frozen PC in 6% DMSO (n = 5).

<table>
<thead>
<tr>
<th>Time</th>
<th>Platelet count (x 10^11/\muL)</th>
<th>FTR (%)</th>
<th>MPV (fL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>680.0 (577–742)</td>
<td>68.0 (57.7–74.2)</td>
<td>11.1 (9.6–13.8)†</td>
</tr>
<tr>
<td>T0 + 2 h</td>
<td>509.0 (408–506)†</td>
<td>50.9 (45.6–58.6)†</td>
<td>11.1 (9.7–17.4)†</td>
</tr>
</tbody>
</table>

*Significantly (P < 0.05) different from data at T0. †Significantly (P < 0.05) different from fresh platelets.
platelets activated with thrombin. The number of microparticles, $1,530 \pm 90$, in these preparations also increased, compared with that of untreated fresh platelets in the resting state. Thrombin activation of fresh platelets in 6% DMSO induced a shift in the population that was indistinguishable from thrombin-activated fresh platelets that were not treated with DMSO. Although the difference was not significant ($P = 0.082$), slightly fewer microparticles were produced ($4,530 \pm 1,210$).

Unstimulated platelets from frozen PCs were shifted to lower forward and side scatter values in a manner characteristic of activated platelets. These platelets had a compact distribution, consistent with a uniform population of cells, which did not change further upon stimulation with thrombin. Interestingly, no increase in the number of microparticles was observed in platelets from PC either before or after stimulation with thrombin ($760 \pm 200$ and $840 \pm 200$ [$P = 0.545$], respectively).

**Discussion**

Variability was found in platelet numbers between the units of the commercial DMSO-stabilized frozen PCs as well as loss of platelets during the freeze-thaw process and after thaw. The platelet population in the frozen PC had maximal amounts of phosphatidylserine at rest and after addition of the platelet agonist thrombin. This is consistent with the presence of an activated platelet population in the frozen PC at thaw.

Prior studies evaluating frozen canine PC reported platelet concentration corresponding to $1.6$ to $5 \times 10^{11}$ platelets/100-mL bag after thaw. In veterinary medicine, there is currently no universally accepted definition of platelet numbers in a PC. The manufacturer of the commercial frozen PC indicated that the initial PC was $1.0 \times 10^{11}$ platelets/100-mL bag, similar to the definition used in human medicine. In our study, the median platelet count in PCs was $5.90 \times 10^{10}$ platelets/100-mL bag, corresponding to a 59% recovery rate, consistent with the previously published literature. The veterinary and human literature report a recovery of 60% to 70%, depending on storage temperature, duration of storage, and blood collection procedure.

The term platelet storage lesion is used in human medicine to describe structural, biochemical, and functional alteration of platelets caused by platelet product collection, preparation, and storage. Storage lesions are influenced by several factors such as mechanical manipulation during platelet apheresis or centrifugation, storage conditions (eg, cooling at 4°C or freezing methods), storage containers, and the use of DMSO (the most commonly used cryopreservative for human and canine platelets). Those changes are considered deleterious and are the hallmark of platelet activation.

Increases in platelet volume, as we found in our study, have been used as an indicator of platelet storage lesions. Because of the technology used for size measurement, increased MPV can be caused by platelet microparticles, cell swelling, shape change (from discoid to spherical), or extension of pseudopods and can represent platelets with a longer lifespan. Increased MPV and irreversible loss of discoid shape are associated with increased reactivity and can be in-

![Figure 1—Detection of phosphatidylserine on canine platelets (fresh with or without 6% DMSO or cryopreserved in 6% DMSO) with or without treatment with thrombin (1 U/mL). Each panel shows overlaid histograms of FITC-labeled annexin V bound to platelets. A—Comparison of fresh platelets in the resting state (shaded histogram) and fresh platelets activated with thrombin (dashed line histogram). B—Comparison of fresh platelets in the resting state (shaded histogram) with fresh platelets in 6% DMSO in the resting state (solid line histogram) and fresh platelets in 6% DMSO activated with thrombin (dashed line histogram). C—Comparison of fresh platelets in the resting state (shaded histogram) with platelets from a commercial frozen PC in the resting state (solid line histogram) and platelets from a commercial frozen PC after activation with thrombin (dashed line histogram).](image-url)
Figure 2—Size distributions of platelets (fresh with or without 6% DMSO or cryopreserved in 6% DMSO) with or without treatment with thrombin (1 U/mL). Each panel depicts the forward (Log FSC) and side scatter (Log SSC) distributions of platelets. Panels correspond to fresh platelets in the resting state (A), fresh platelets activated with thrombin (B), fresh platelets in 6% DMSO (C), fresh platelets in 6% DMSO activated with thrombin (D), unstimulated platelets from commercial frozen PC (E), and platelets from commercial frozen PC activated with thrombin (F). All platelets were loaded with 1mM CaCl₂ prior to subsequent analysis or activation, which was performed with thrombin at 1 U/mL. Distributions are color coded according to a logarithmic scale corresponding to the number of platelets at each position in the diagram; blue has the minimum number of platelets and red the maximum. The encircled distribution near the origin of the plot in panel A corresponds to the microparticle population.
duced by prolonged cold (4°C) storage. Microvesiculation, or the generation of microparticles, is associated with storage lesions and is considered a marker of activation. Furthermore, these platelet microparticles are reported to have greatly increased procoagulant activity. Flow cytometric analysis of the frozen PC revealed an increase in the number of microparticles, as well as smaller platelets. As noted, platelet microaggregates may lead to a general increase of MPV in the product.

Platelet storage lesions also are characterized by accumulation of lactic acid and acidosis, with a pH below 6.2 associated with decreased in vivo survival. Aging of the PC is associated with decreased Pco2 and glucose content in both human and veterinary studies. The acid-base status of the studied PCs is consistent with the use of citrate-phosphate-dextrose-adenine as the anticoagulant and storage agent.

In our study, the Pco2 and Pco2 were higher in the platelet storage container than in room air (176 mm Hg, compared with 160 mm Hg in room air, and 37.9 mm Hg, compared with 0.25 mm Hg in room air, respectively) and may be a sign of decreased gas exchange between the PC and the environment, which is highly dependent on the plasticizer used. Plasticizers are chemical components added to the hard plastic bag to improve its flexibility and durability. Each one has innate chemical and physical characteristics that modify gas exchange through the collection system.

Loss of cellular function associated with room temperature platelet storage lesions has been reported in the human literature. Increased amounts of phosphatidylserine or P-selectin on the platelet surface, decreased aggregometry response to agonist, or decreased recovery after hypotonic stress reaction are all evidence of a loss of platelet function. In our experiment, the platelets in the frozen concentrate already had maximal amounts of phosphatidylserine at rest, compared with fresh platelets. Moreover, platelets in the frozen concentrate are not able to translocate additional phosphatidylserine to the outer leaflet of the plasma membrane when stimulated with thrombin, whereas the fresh platelets and the fresh platelets in 6% DMSO could. In fresh platelets, with or without 6% DMSO, a portion of the population (33% and 16%, respectively) remained unactivated, even in the presence of thrombin, because the concentration used in these experiments (1 U/mL) is insufficient to induce translocation of phosphatidylserine in 100% of the fresh platelet population. However, the concentration of thrombin used in this experiment is closer to physiologic amounts than that which would be required for 100% translocation of phosphatidylserine.

In conclusion, findings in our study revealed that the commercial frozen PC had a decreased platelet number after thaw. The flow cytometry analysis revealed that the platelets in the frozen concentrate already had maximal amounts of phosphatidylserine and, when stimulated with thrombin, were unable to translocate additional phosphatidylserine to the outer leaflet of the platelet plasma membrane as required for platelet activation.

References