Evaluation of darbepoetin therapy on platelet count and function in healthy cats and cats with surgically induced chronic kidney disease

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OBJECTIVE
To evaluate the effects of darbepoetin on platelet population and reactivity in healthy cats (HCs) and azotemic cats with remnant kidney (RK) model–induced chronic kidney disease.

ANIMALS
12 purpose-bred domestic shorthair cats (n = 6 HCs and n = 6 RK).

METHODS
In this pilot study, all cats received darbepoetin (1 µg/kg, SC) on days 0, 7, and 14. Blood was sampled at baseline and on days 3, 10, 15, 17, 20, and 21. At each time point, a CBC was performed, platelet aggregometry was assessed by impedance and optical methods, and platelet P-selectin (CD62P) was quantified before and after thrombin stimulation. Additionally, reticulated platelets were quantified using both thiazole orange staining and proprietary analysis by the CBC analyzer. For RK cats, systemic blood pressure (BP) was serially measured.

RESULTS
No adverse effects of darbepoetin were seen. There was no statistically significant change in platelet count between or within groups at any time point. Hematocrit increased significantly over time in the RK but not the HC group. RBC reticulocyte numbers in both groups increased over time. Reticulated platelet percentage did not increase in either group. Differences in platelet reactivity within or between groups were not seen in the aggregometry or flow cytometric assessments. In RK cats, indirect BP did not significantly change during the study.

CLINICAL RELEVANCE
This preliminary investigation did not find evidence that darbepoetin administration impacted platelet number, reactivity, nor reticulated platelet count. Anemic RK cats experienced increased hematocrit and RBC reticulocytes as expected with darbepoetin therapy.

Keywords: veterinary, chronic kidney disease, erythropoietin, nephrology, feline

Darbepoetin alfa (DPO) is a hyperglycosylated recombinant human erythropoietin (EPO) analogue developed to provide an increased half-life and longer duration of action compared to other forms of exogenous EPO. Both analogues act on erythroid progenitor cell receptors in the bone marrow to stimulate erythropoiesis and increase Hct. In human and veterinary medicine, DPO is commonly given to patients with chronic progressive anemia, including those with anemia secondary to chronic kidney disease (CKD).

Although EPO and DPO effectively increase Hct, there is an increased risk of thromboembolic events in humans receiving therapy with either drug. In a meta-analysis of 9 randomized, controlled clinical trials, humans with CKD receiving EPO injections targeting a high hemoglobin concentration had a higher risk of dialysis-access thrombosis (risk ratio, 1.34; 95% CI, 1.16-1.54). In a more recent review in people with anemia secondary to cancer, the overall risk of thromboembolic complications increased by 52% when people were treated with EPO analogues.

Platelet activation following EPO administration, as...
assessed by increased thromboxane B2 and platelet E-selectin, P-selectin (CD62P), and CD63 expression, has been documented in humans. In veterinary medicine, increased platelet reactivity or hypercoagulability can be of particular concern in the context of kidney transplantation, where graft thrombosis can contribute to morbidity and transplant failure.

In a retrospective study of 22 dogs treated with DPO for the management of anemia secondary to CKD, 86% of dogs experienced an increase in platelet count, of which 18% were above the reference interval. In that report, no thromboembolic complications were reported. Another study in healthy adult dogs receiving EPO documented a significant increase in the number of circulating reticulated platelets (approx 10%) as well as increased platelet reactivity.

Although EPO and DPO are commonly used as part of comprehensive treatment of cats with CKD, there is no data regarding the impact of DPO administration on platelet function or reactivity in cats. This study aimed to evaluate the effects of DPO therapy on platelet function and type and markers of platelet reactivity in a cohort of healthy cats (HCs) and another part of comprehensive treatment of cats with CKD, 86% of dogs experienced an increase in platelet count, of which 18% were above the reference interval. In that report, no thromboembolic complications were reported. Another study in healthy adult dogs receiving EPO documented a significant increase in the number of circulating reticulated platelets (approx 10%) as well as increased platelet reactivity. Among the 22 dogs treated with DPO, 21 were characterized as HCs based on the presence of persistent anemia (Hct < 35) and suitable temperament.

Methods

Animals
All study activities were approved by the University of Georgia IACUC (Animal Use Protocol A2020 12-005-Y1-A3) and followed American Association for Laboratory Animal Science guidelines for Humane Care and Use of Laboratory Animals. Twelve adult, purpose-bred cats were used in the study. Six were characterized as HCs based on physical examination and baseline serum biochemistry profile, CBC, and urinalysis. Six additional cats with CKD caused by selective arterial ligation and contralateral nephrectomy as part of a remnant kidney (RK) model were also studied. The surgeries to create the RK model occurred approximately 2 years prior to the study described here. A more complete description of the colony has been previously published.

Cats were selected for inclusion in the RK group based on the presence of persistent anemia (Hct < 5%) and suitable temperament.

Study treatments
This study was designed as a prospective pilot study without masking of the therapeutic regimen. DPO (Aranesp; Amgen Inc) was administered to all cats subcutaneously in the area between the scapulae with a 27-gauge needle at a dose of 1 µg/kg once every 7 days (days 0, 7, and 14). Iron dextran was also given to each cat in the RK group (50 mg/cat, IM) on the day of their baseline measurements.

Blood collection
All cats were fasted at least 12 hours prior to venipuncture. Blood was drawn on days 3, 10, 15, 17, 20, and 21 for all groups, with an additional sample point taken as baseline in the RK group, designated as day 0. Baseline CBC and impedance aggregometry data was also collected from the HCs at day 0, but platelet-rich plasma (PRP) was not prepared to evaluate optical aggregometry. Cats were sedated for all blood draws with ketamine (7 mg/kg, IM), midazolam (0.2 mg/kg, IM), and dexametomidine (5 mg/kg, IM). One cat developed severe bradycardia and hypotension following this combination on the first sample day and did not receive dexametomidine for subsequent sedations. Two cats vomited after their first sedation event and subsequently received an oral dose of maropitant at an approximate dose of 1 mg/kg the night before sedation.

During blood collection, the cats were placed in lateral recumbency, and 6 mL of blood were sampled from a jugular vein using a 22-gauge needle and 3- or 6-mL syringes. Blood was immediately transferred to appropriate blood tubes containing no additive, 3.2% sodium citrate (in a 1:9 citrate:blood ratio), and EDTA. All subsequent assays were completed within 4 hours of blood collection. After removal of a 1-mL aliquot of whole blood for impedance platelet aggregometry, the citrated samples (2 mL) were processed into PRP through centrifugation at 200 X g for 10 minutes after resting upright at room temperature (24 °C) for 30 minutes. After removal of PRP, the remaining sample was centrifuged at 1,200 X g for 10 minutes to harvest the supernatant as platelet-poor plasma.

Complete blood count and platelet analysis
CBC, including platelet parameters and reticulated platelet count, was performed on the EDTA-anticoagulated sample using proprietary software and automated sample preparation (ADVIA 2120 Hematology system; Siemens).

Impedance and optical aggregometry
Impedance platelet aggregometry (Model 700 Lumi-aggregometer; Chrono-log Corp) was performed between 30 minutes and 1 hour of blood collection. Briefly, 500 µL of citrate-anticoagulated whole blood was incubated with 500 µL of 0.9% NaCl at 37 °C with continuous stirring (1,200 rpm). Aggregation assays were performed using 40 µM ADP (Chronolog Corp) in 1 cuvette and 10 mg/dL collagen (Chronolog Corp) in the other. The change in impedance, reflecting platelet aggregation, was recorded for 6 minutes using commercially available software (Aggrolink; Chronolog Corp) that reported the extent of aggregation (in ohms) and the area under the aggregation curve.

Optical aggregometry was also performed when there was adequate sample available. PRP was diluted with platelet-poor plasma to a final platelet count between 250 and 300 X 10^9 platelets/µL and placed into 2 siliconized glass cuvettes with continuous stirring (1,200 rpm) at 37 °C. Aggregation was induced with ADP (40 µM) in 1 cuvette and collagen (10 mg/dL) in the other cuvette and observed for 6 minutes. The percentage of aggregation as a function of light
transmittance was recorded (Aggrolink; Chronolog Corp).

**Flow cytometry**

Platelet integrin B3 (CD61) was identified using a phycoerythrin (PE)-conjugated mouse anti-human antibody (CD61-PE; clone VI-PL2; Invitrogen), and platelet activation was identified through CD62P expression using an Alexa Fluor 647 (A647)-conjugated rat anti-mouse antibody (clone RB40.34; BD Pharmingen). Platelet activation was assessed in both resting platelets and in platelets that were activated in vitro using bovine α-thrombin (Sigma Aldrich). An isotype-matched control antibody (rat IgG1-A647, clone A110–1; BD Pharmingen) was used to verify specific binding of CD62P. Reticulated platelets were identified using thiazole orange (TO) binding (UltraPure Grade AS; AnaSpec) coupled with forward scatter gating.\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)

An aliquot of PRP was diluted to a final concentration of 1 X 10\(^4\) cells/μL using a modified Tyrode’s buffer (pH 7.2, 12 mM NaHCO\(_3\), 138 mM NaCl, 5.5 mM dextrose, 2.9 mM KCl, 10 mM HEPES)\(^3\)\(^4\) with 20 mM Gly-Pro-Arg-Pro amide (Sigma Aldrich) before stimulation with thrombin (final concentration, 2 U/mL) or buffer (negative control) for 15 minutes. Antibodies for CD61 and CD62P were sequentially incubated with the platelets for 20 minutes at room temperature (24 °C) in the dark. Subsequently, samples were incubated in the dark with TO (0.35 μg/mL) for 30 minutes at 24 °C. After antibody labeling, 150 μL of modified Tyrode’s buffer was added, and samples were analyzed using a NovoCyte Quaneeon flow cytometer with NovoExpress software (Agilent Technologies) within 1 hour of preparation.

Flow cytometric data was collected as 10,000 putative single platelets per sample. A digital compensation matrix including all fluorochromes was established and applied during data acquisition to compensate for spectral overlap. Platelets were identified using anti-CD61-PE and forward (diffracted) and side (refracted) light scatter and a gate/threshold combination set to acquire the maximum number of platelets while minimizing noise and debris (Supplementary Figure S1). Data were analyzed using commercial software (FlowJo Software, version 10.8.1; BD Biosciences).

Mature and immature/reticulated platelets were identified using a previously established gating strategy including both forward scatter and TO staining.\(^3\)\(^4\) This gating strategy was applied to correct for the increased size of reticulated platelets relative to mature platelets affecting their fluorescence (Supplementary Figure S2). A threshold of positivity for CD62P staining was defined based on the fluorescence of the isotype-A647–labeled platelets (< 1%). This threshold was confirmed as biologically relevant when compared to the data from the control (unactivated) samples.

**Blood pressure**

Indirect systemic arterial blood pressure (BP) was measured in all RK cats using a Doppler ultrasonic flow probe and sphygmomanometer (Series DS66 Trigger Aneroids; Welch Allyn) in accordance with American College of Veterinary Internal Medicine recommendations.\(^1\) At the time of the study, no cat with CKD was hypertensive or receiving antihypertensive medication. Cats with surgically induced CKD were selected for inclusion in the RK group of the present study based on the presence of persistent anemia and suitable temperament. Indirect BP was measured at days 1, 8, 16, and 23 for cats in the RK group, which were accustomed to awake BP measurement as it was a routine part of their CKD monitoring prior to the start of this study. Longitudinal monitoring of BP was not performed in the HC group.

**Statistical analysis**

All analyses were performed using commercial statistical software (SAS, version 9.4, SAS Institute Inc; SPSS Statistics, IBM Corp; and Prism, version 8.4.3, GraphPad Software LLC). Values of \(P < .05\) were considered statistically significant. CBC data for both groups and BP data from the RK group were compared within groups using a repeated measures ANOVA or a mixed effects model, with the Tukey test applied for multiple comparisons. Differences between stimulated and unstimulated values were calculated for each cat prior to the analysis of flow cytometry data. Linear mixed models were used for analyses of aggregometry, flow cytometry stimulation differences (ie, magnitude of change from stimulated to unstimulated), and CBC values between groups. Linear mixed models were developed for each variable separately that included fixed factors of group, day, and a group-by-day interaction factor, and a random intercept for each cat was included to account for within-cat correlation. Histograms and quantile-quantile plots of conditional model residuals were examined to evaluate the assumption of normality. Plots of residuals and conditional residuals versus predicted values of measurements were examined to evaluate the assumption of homogeneity of variances. CD61 + TO + flow cytometry differences had nonhomogeneous variance, so Welch \(t\) tests for each day separately were used. Some CBC values were log transformed prior to analysis to make the assumption of homogeneity of variances valid. Simple tests between groups for each day and between days for each group were performed. Group comparison \(P\) values were adjusted for multiplicity using the adaptive linear step-up false discovery rate method of Benjamini and Hochberg\(^2\) for each variable separately. The Satterthwaite degrees of freedom method was used to adjust the error degrees of freedom for multiple variances, which maintains type I error rates at the purported levels. Restricted maximum likelihood estimation was used to reduce bias in variance estimates. Data are expressed as mean ± SD unless otherwise noted.

**Results**

The 6 cats in the HC group were all neutered males aged 2 to 3 years and weighing 5.98 ± 1 kg. Of the RK cats, 3 were spayed females, and 3 were...
neutered males. RK cats were between 3 and 4 years of age and weighed 4.83 ± 0.6 kg. All RK cats had International Renal Interest Society (IRIS) CKD stage 2 or 3 kidney disease, characterized by a serum creatinine concentration greater than 1.6 mg/dL and a serum symmetric dimethylarginine concentration greater than 18 µg/dL. No adverse effects related to the administration of DPO were observed during the study period.

**Blood pressure**

The average baseline BP for the RK group was 138.2 mm Hg ± 17.3 (range, 116.8–163.2 mm Hg). At baseline, 3 cats were normotensive (BP < 140 mm Hg), 2 cats were pre-hypertensive (BP, 140–159 mm Hg), and 1 cat was hypertensive (BP, 160–179 mm Hg), according to the categories set forth by the American College of Veterinary Internal Medicine. Over the 3 weeks of the study, BP in the RK cats did not significantly change (P = .22), although large intra- and interindividual variability was noted. Only 2 cats had BP readings in the hypertensive range during DPO treatment (1 cat of which was normotensive and 1 cat hypertensive at baseline), none of which remained hypertensive at study completion.

**Complete blood count analysis**

The HC group had a mean baseline Hct of 34.9% ± 2.6 (range, 30.7% to 37.5%), whereas the RK group had a baseline Hct of 28.7% ± 3.7 (range, 23.8% to 32.8%).

![Figure 1](image1.png)  
Figure 1—Percentage of hematocrit (circles) as compared to percentage of reticulated red blood cells (squares) in healthy cats (A) and cats with chronic kidney disease (B) receiving darbepoetin alfa injections on days 0, 7, and 14. Error bars reflect SD. *Values different from the baseline value (P ≤ .04). #Values different from the day 3 value (P ≤ .04).

![Figure 2](image2.png)  
Figure 2—Aggregation responses to ADP (A and B) and collagen (C and D) for both impedance (squares, right vertical axis) and optical (circles, left vertical axis) aggregometry. Data from healthy cats is summarized in (A) and (C) and cats with chronic kidney disease in (B) and (D). Error bars reflect SD.
Cats in the RK group had a mean baseline serum creatinine concentration of 2.2 mg/dL ± 0.45 (range, 1.9 to 3.1 mg/dL). Hematocrit was significantly higher in the HC group than the RK group on days 0 and 3 (P = .014 and 0.037, respectively) but not on subsequent days. The Hct increased in the RK group and was significantly higher than the day 0 Hct on days 15, 17, and 20 (P = .001, 0.003, and 0.003, respectively) and significantly higher than day 3 on days 15 and 20 (P = .021 and 0.034, respectively; Figure 1). There was no significant change during the study in the Hct of the HC group (P = .1523; Figure 1). In the RK group, the RBC reticulocyte percentage was significantly increased on day 10 compared to day 0 (P = .038; Figure 1). While the day-to-day difference in the total RBC reticulocyte count in the RK group was initially significant (P = .020), it failed to meet significance criteria for multiple comparisons, and no individual day was significantly different than another. The RBC reticulocyte percentage in the HC group was increased above day 0 on days 3, 17, and 21 (P = .012, 0.040, and 0.017, respectively; Figure 1), and the total RBC reticulocyte count increased in the HC group between day 0 and day 3 and between day 15 and day 17 (P = .024 and P = .0425, respectively). The HC group had a mean baseline platelet count of 365.8 ± 98 X 10^3/μL (range, 181 to 468 X 10^3/μL), whereas the RK group had a baseline Hct of 348.5 ± 100 X 10^3/μL (range, 192 to 479 X 10^3/μL). In both groups, there were no statistically significant changes in platelet count within groups over the course of the study (P = .840 and 0.110 for HC and RK groups, respectively). There were no significant differences in other parameters of the CBC between either group, including reticulated platelet parameters.

Impedance and optical aggregometry

For optical aggregometry, there was no difference in the maximal aggregation response to ADP in either group or between groups for the duration of the study (P = .970 for HC, P = .980 for RK, and P = .980 for group comparison; Figure 2). There was also no difference in the maximal aggregation response to collagen in either group or between groups for the duration of the study (P = .056 for HC, P = .066 for RK, P = .238 for group comparison; Figure 2). Impedance aggregometry, compared as area under the aggregation curve, was not statistically different at any time point for ADP-induced aggregation (P = .991 for HC, P = .160 for RK, and P = .214 for group comparison; Figure 2). For collagen-induced aggregation...
impedance aggregometry, there was also no difference between groups or within groups at any time point (P = .997 for HC, P = .785 for RK, and P = .953 for group comparison; Figure 2).

**Flow cytometry**

There was no difference between groups or with time in CD61 expression with and without thrombin stimulation (P = .935). In the HC group, the percentage of TO expression in the CD61-positive gate did not change with time or with stimulation (P = .873 unstimulated, P = .139 stimulated; Figure 3). The same was true in the RK cats (P = .850 unstimulated, P = .982 stimulated; Figure 3). There was no difference in TO expression on any day between the HC and RK groups (P > .982). There was no difference between groups or within groups at any time in CD61 expression with and without thrombin stimulation (P = .935). CD62P = P-selectin.

**Discussion**

Weekly dosing of DPO given subcutaneously over 3 weeks at a dosage of 1 µg/kg in HCs and anemic cats with CKD did not have detectable effects on platelet number nor platelet reactivity. A therapeutic effect of DPO was observed in the RK cats, which demonstrated an increase in Hct and RBC reticulocytes from baseline, but we did not demonstrate an increased Hct in the HC group despite increased numbers of RBC reticulocytes.

Reticulated platelets are immature platelets with a high dense granule content, and increased circulating reticulated platelet numbers can be an independent predictor of cardiovascular ischemic events in humans. We hypothesized that the general bone marrow stimulation caused by the administration of DPO would also increase the release of reticulated platelets and may contribute to a procoagulant state through a shift in the platelet population toward a more reactive one. Although we were able to show that the unstimulated reticulated platelets were expressing CD62P to a greater degree than mature platelets, we were unable to definitively demonstrate that their presence affected platelet aggregation. Although the reticulated platelets were circulating at a higher activation state, they responded to thrombin exposure to the same degree (as measured by CD62P expression) as did the mature platelets.

We assessed the number of reticulated platelets using 2 different methodologies: the estimates derived from the ADVIA 2120 CBC analyzer and TO staining and flow cytometry. The percentages of reticulated platelets calculated by each method were not different, but we failed to demonstrate a significant correlation between the 2 methods. These results are similar to a recent report in dogs comparing the 2 methods. Despite the lack of significant correlation between the 2 measures, the lack of difference between the 2 in a paired analysis suggests that either method may give a general idea of the presence of reticulated platelets in a given sample.

This study attempted to document changes in platelet population and reactivity using different techniques and approaches. Impedance aggregometry and optical aggregometry both assess the ability of platelets to physically respond to specific stimuli by causing platelet shape change and aggregation under low-shear conditions. Both techniques were used because impedance aggregometry requires no additional processing of citrated whole blood, whereas optical aggregometry requires the production of PRP, which may theoretically activate the platelets during processing and provide a source of error. Flow cytometry is another low-shear environment that (as tested here) investigates the ability of the platelets to respond to a stimulus through the externalization of alpha granule contents, indicated by the translocation of CD62P to the platelet surface. One human study documented higher concentrations of soluble CD62P in patients who received EPO treatment undergoing a single hemodialysis treatment compared to those who did not.
increased soluble CD62P was thought to represent an increased activation state of platelets in the EPO-treated group such that the dialysis session triggered more platelet activation and CD62P release. The administration of DPO did not change the platelet response to any stimulus at any timepoint in the current study, but we did not provide as provocative an stimulus as extracorporeal therapy.

Human patients receiving EPO experience a higher risk of dialysis-access thrombosis, higher rates of major cardiovascular events, and an increased risk of thromboembolism. Although human studies have suggested increased platelet and endothelial reactivity following EPO treatment, no underlying mechanisms have been elucidated. The impact of increased RBC mass alone on coagulation must also be considered. In humans, elevated Hct is associated with myocardial infarction and venous thromboembolism. This may be due to factors such as blood viscosity, increased contact of the platelets with the endothelial surface, and the release of platelet-activating factors such as hemoglobin and ADP released from damaged RBCs. The focus on platelet function in the current study may have missed an overall shift toward hypercoagulability occurring in the DPO-treated cats.

In veterinary medicine, DPO is most commonly used for the treatment of anemia of CKD. Kidney-driven anemia is caused by several contributing factors, including the loss of EPO-producing cells within the damaged kidney, a decrease in erythropoietin level, an impaired bone marrow response inhibiting efficacious iron use for the production of RBCs. In cats with CKD, DPO therapy improves morbidity and mortality. DPO dosage of 1 µg/kg, SQ, given once weekly has previously been studied to have a 56% response rate in cats (compared to 23% of human patients). Regardless of increase in reticulated platelet counts with DPO treatment may imply that cats are not as sensitive to the stimulatory effects that have been reported in dogs. In the more specific context of EPO therapy and postoperative thrombosis, increased reticuloocytes combined with elevated postoperative fibrinogen may create a more effective procoagulant atmosphere, and assays of global hemostasis (eg, thromboelastography) may better shed light on this particular question.

Although we performed serial examinations of the cats in this study, overall, the sample size was small, which increases the likelihood of a type II error for all comparisons. That is to say that the possibility of detecting a meaningful increase in platelet reactivity was low. The data provided herein provides guidance for sample sizes in future studies. An assumption was also made that the anemia and CKD as created by the RK model mimics naturally occurring CKD, but this does not account for variability in comorbidities seen in humans with CKD and uremia show evidence of platelet dysfunction. This may occur secondary to an imbalance among platelet agonists, a decreased platelet release of ATP secondary to uremia, or a dysregulation of blood calcium. Among veterinary species, few studies have documented baseline platelet dysfunction in CKD, and most of this literature is described in dogs with CKD. In the current study, there was no difference in the platelet activation potential or circulating activation state between the 2 groups, but the NK animals were not considered to be uremic. Chronic systemic arterial hypertension may also impact platelet reactivity. However, in this study, hypertension was uncommon in NK cats, affecting only up to 2 cats during treatment, and so it is unlikely that it impacted platelet activation state.

Systemic arterial hypertension is the most common comorbidity seen in humans with CKD and has also been reported as a complication secondary to EPO-derivative therapy. In veterinary medicine, hypertension is reported as an adverse event to EPO administration in 40% of dogs and 50% of cats (compared to 23% of human patients). The cause of elevated BP secondary to EPO derivatives is unknown and is likely multifactorial, but the most likely theory revolves around increased blood viscosity due to alterations in red cell mass. In the present study, we found no statistically significant difference in indirect BP over time for the CKD cohort despite DPO therapy and documented increases in Hct. Unfortunately, the HC group did not undergo regular BP monitoring, so the impact of DPO therapy on BP in this cohort cannot be determined.
in this study cohort. Additional studies in specific populations of interest (eg, naturally occurring CKD of advanced IRIS stages, postoperative kidney transplant patients) are indicated to delineate the specific impacts of DPO on those populations.

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Disclosures

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org.