

# Clinical and Hematological Effects of Serial Phlebotomy Performed on Laboratory Beagles

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We conducted a study designed to mimic a typical pharmacokinetic study to gain a better understanding of a dog's response to multiple, frequent blood sampling at 15% total blood volume. Ten dogs were randomly assigned to either a control group having sham venipuncture performed or to a blood collection group having 1.5% of their body weight (approximately 15% total blood volume) removed weekly for 4 weeks. Both groups were monitored during a 2-week recovery period immediately after the 4-week collection period. Parameters evaluated were clinical signs, body weight, and hematological and serum biochemical analytes. There were minimal differences in red blood cell morphology between the two groups. Statistically significant differences in hematocrit between the two groups occurred on several days, and this finding was attributed to blood withdrawal in the blood collection group; however, this statistical difference was not deemed to be clinically significant. There were no statistically significant differences in body weight, total protein, reticulocyte count, mean corpuscular volume, mean corpuscular hemoglobin, or red cell distribution width. We conclude that removing 15% blood volume in laboratory beagles is compatible with maintaining the health and well-being of the dog and can be acceptable in laboratory situations when it is scientifically justified.

Serial phlebotomy is a common practice in biomedical research in which laboratory animals are used. Pharmaceutical research relies on serial phlebotomy in toxicokinetic, pharmacokinetic, and drug metabolism studies to provide an appropriate volume of blood at predetermined times for drug concentration analyses. As an example, a typical pharmacokinetic study regimen involves dosing an animal at time 0, then withdrawing blood samples at 0, 2, and 30 min and 1, 2, 4, 6, 8, 12, 24, and 48 h after dosing. If a large amount of blood is removed too rapidly, too frequently, and/or without volume replacement, the animal may be stressed and unfavorable experimental variables may occur, including extreme physical stress, hypovolemic shock, and anemia (1-3).

Researchers have found that removing as little as 10% of the circulating blood volume of an animal at one time may be enough to initiate cholinergic mechanisms to restore homeostasis (1, 4). When 15% to 20% of the blood volume of an animal is removed at one time, cardiac output and blood pressure may be reduced, and when 30% to 40% is removed, hemorrhagic shock and mortality has occurred in rats and other species (3-5). Whenever blood is collected, the phlebotomist must monitor for early signs of hypovolemic shock and anemia and prompt veterinary medical attention afforded if complications develop. Signs indicating shock and anemia include pale mucous membranes, increased respiratory effort, and exercise intolerance (1, 4, 5).

Recommendations for total blood collection limits in dogs and other species are often based empirically on previous blood collection studies, personal experience, animal welfare concerns and/or an investigator's research purposes (4, 6, 7). One recommendation for good laboratory practice is to limit total blood collection in dogs to no more than 10% of the circulating blood volume every 2 weeks (1, 4, 8). However, while a new compound is being tested in a pharmaceutical setting, investigators may be required to take multiple small blood samples from the same dog in a timed fashion during a 24- to 48-h time period, thereby potentially exceeding this recommendation and sometimes totaling 15% or more of a dog's circulating blood volume.

We sought to determine the clinical signs and hematological effects of removing 15% of the circulating blood volume from beagles, repeating the procedure once a week for 4 weeks, and then evaluating the dogs during a 2-week recovery period. Throughout the study period we monitored all clinical signs exhibited and routinely checked blood parameters.

## Materials and Methods

**Study subjects.** All dogs used during this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* at Pfizer Corporation, Kalamazoo, Mich., an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-accredited facility. All procedures were reviewed and approved by Pfizer Corporation's Institutional Animal Care and Use Committee. The study population comprised 6- to 8-month-old male and female purpose-bred beagles (*Canis familiaris*; Covance Research Products, Inc., Denver, Pa.). Animals were housed in a controlled environment in individual runs on aspen shavings (Northeastern Products, Warrensburg, N.Y.) as direct-contact bedding. The acclimation period prior to the study was 2 weeks. Dogs were limit-fed 300 g of a complete diet (Certified Canine Diet #5007, PMI Feeds, Inc., St. Louis, Mo.) once daily and provided with potable chlorinated deionized water ad libitum from an automatic watering system. They were housed in a conventional animal room with 100% fresh conditioned air at 36.1 to 41.6°C (65 to 75°F) and 30% to 70% humidity, with 12 air changes per hour and a 12:12-h light:dark cycle.

**Study design.** Ten beagles (five females and five males) were randomly assigned to two groups; five to a control group and five to a blood collection group. The control group consisted of three male and two female dogs. The blood collection group consisted of two male and three female dogs. The blood volume to collect for the blood collection group was calculated based on the historically derived "10% rule," which states that 10% of an animal's body weight is blood volume, thus 1% of an animal's body weight would equal 10% of total blood volume (1, 4). In our experiment, we extrapolated to assume 1.5% of the animal's body weight would equal 15% of total blood volume. The blood was collected via jugular venipuncture. The control group experienced jugular venipuncture without

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**Table 1.** Calculated blood removal volume based on body weight

Weight of dog (kg)	Total volume (ml) removed <sup>a</sup>	Volume (ml) removed at each of four collection times on day 1 <sup>b</sup>
8.75–8.99	131.3	28.8
9.00–9.24	135.0	29.8
9.25–9.49	138.8	30.7
9.50–9.74	142.5	31.6
9.75–9.99	146.3	32.5
10.00–10.24	150.0	33.5
10.25–10.49	153.8	34.4
10.50–10.74	157.5	35.4
10.75–10.99	161.3	36.3
11.00–11.24	165.0	37.3
11.25–11.49	168.8	38.2
11.50–11.74	172.5	39.1
11.75–11.99	176.3	40.1
12.00–12.24	180.0	41.0
12.25–12.49	187.5	42.9
12.50–12.74	191.3	43.8

<sup>a</sup>The total volume removed represents 15% of total blood volume, which is assumed to be 10% of total body weight.

<sup>b</sup>Total volume removed at each collection time on day 1 of each week; value accounts for removal of 16 ml of blood for hematology later in the week.

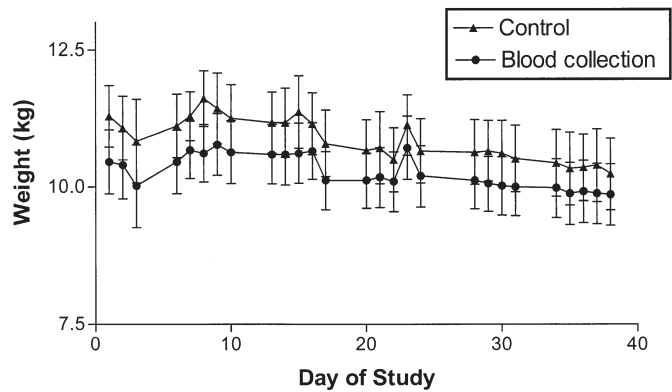
blood collection (except for the collection of 8-ml samples for hematological analysis, as described below).

**Blood collection technique.** Jugular venipuncture was performed using a 23-gauge needle and a 12-cc syringe. Hair over the venipuncture site was removed with a #40 clipper, and the skin was prepped with alcohol. Food was withheld from each dog overnight (approximately 16 h) prior to and until 3 h after phlebotomy, in the manner of a typical pharmacokinetic study. Each dog was weighed immediately before blood collection, and the total volume to collect was calculated by rounding down the weight to the nearest quarter kilogram, multiplying by 0.015, and subtracting 16 (representing the total volume of blood in ml to be obtained for hematological analyses conducted later in the week). The total volume was then divided by 4, representing the volume to be collected during each of four separate time periods equally distributed during a 9-h period (Table 1). Each sample was divided among a 3-ml potassium EDTA glass container, 2-ml 3.8% sodium citrate glass container, and 3-ml no-additive glass container (Becton Dickinson Vacutainer Systems, Franklin Lakes, N.J.) and submitted for analyses.

**Experimental conditions.** The experiment was divided into two phases: a 4-week blood volume collection phase and a 2-week recovery phase.

For the blood collection group, the 4-week phase involved removing blood at times 0, 3, 6, and 9 h on days 1, 8, 15, and 22 of the study. Additional blood was collected for laboratory analysis on day 3 and 6 of each week. For the control group during the first 4 weeks on day 1 at time 0, 8 ml of blood was collected for hematological analysis. At 3, 6, and 9 h, jugular venipuncture was done to simulate the act of phlebotomy, but no blood was collected. However, 8-ml samples were collected on the third and sixth day of each week for laboratory hematological analyses as described previously. For both groups of dogs, the collection phase was followed immediately by a 2-week recovery period during which 8-ml samples were collected for laboratory analyses on days 1, 3, and 6 of each week. Hematological analyses included complete blood count, reticulocyte count, total protein, and coagulation assays.

Behavioral and health observations were made at each sampling time and at least daily by study personnel and/or animal care technicians. Body weights were obtained daily (except weekends and one



**Figure 1.** Body weight (mean  $\pm$  standard error of the mean) over time.

holiday day). Signs interpreted as indicative of pain or distress included dull eyes or hair coat, decreased skin turgor, abnormal heart rate or rhythm (lasting for > 3 sec), abnormal color or moistness of mucous membranes, slowed capillary refill time, increased sensitivity to or withdrawal from touch, lack of affiliative response (e.g., tail wagging or affection seeking), vocalization, or decrease in activity. Urine and feces were monitored for abnormalities in volume, frequency, or character.

**Laboratory analyses.** Analyses were performed with an automated cell counter (Abbott Cell-Dyn 3500, Abbott Laboratories, Abbott Park, Ill.). Parameters determined include leukocyte count, differential leukocyte count, hematocrit (Hct), erythrocyte count, hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, and red blood cell distribution width (RDW). MCV and MCH were derived using a machine calibrated specifically for the canine species. Absolute reticulocyte numbers and reticulocyte percentages were measured (Sysmex R3000 Reticulocyte Analyzer, Sysmex Corp., Long Grove, Ill.). A technician evaluated a blood smear for cellular morphology. Coagulation assays performed (Baxter MLA 1000C/900C, Baxter Healthcare Corp., Deerfield, Ill.) include prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen levels. Serum biochemical analysis was performed (Beckman CX7, Beckman Coulter, Fullerton, Calif.), but only total protein was reported.

**Data analysis.** Body weight, clinical observations, and clinical pathology results were entered into an electronic data storage system (Xyber Corporation, Cedar Knolls, N.J.). Each animal was identified by an experimental number in this system. Quantitative data on all animals was entered into a statistical analysis program (SAS, SAS Institute, Inc. Cary, N.C.). The following variables were modeled for statistical significance: hematocrit, body weight, total protein, reticulocyte count, MCV, MCH, and RDW. The model was a mixed one, using each dog in its treatment group (control or blood collection) and day of study. The day of study was nested in the treatment group. Repeated measures were taken for each dog within the control and blood collection groups. These measures were modeled using a heterogeneous first-order autoregressive [arh(1)] correlation matrix. The arh(1) allows for a covariance structure between repeated measures. Power curves were analyzed to determine the likelihood of finding a significant difference in another population when our population had a significant difference. Values were considered significant if they exhibited a power of at least 80% and a  $P$  value  $\leq$  0.05. Data are given as mean  $\pm$  1 standard deviation. Data presented in Fig. 1 through 7 are given as mean  $\pm$  standard error of the mean.

## Results

**Clinical signs and morbidity.** Overall, animals remained healthy

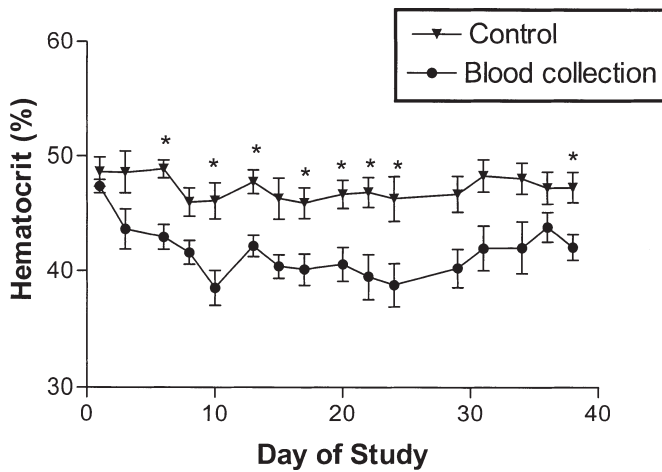


Figure 2. Hematocrit (mean  $\pm$  standard error of the mean) over time. Statistically significant differences between control and blood collection groups occurred on days 6, 10, 13, 17, 20, 22, 24, and 38 (indicated with \*).

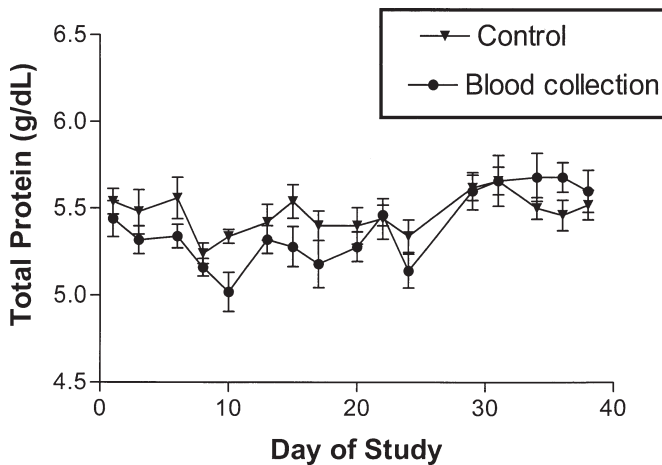


Figure 3. Total protein (mean  $\pm$  standard error of the mean) over time.

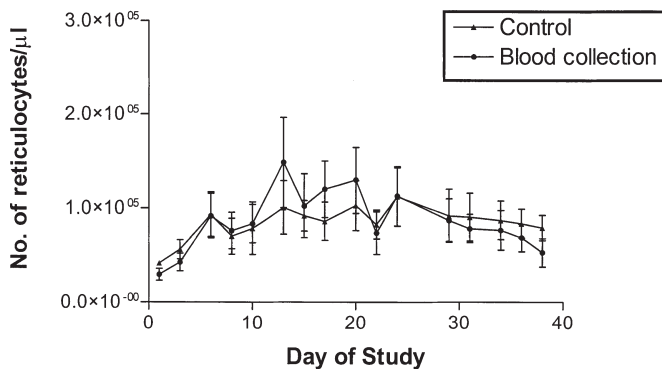


Figure 4. Reticulocyte count (mean  $\pm$  standard error of the mean) over time.

throughout the course of the study. Some clinical signs (i.e., slow capillary refill time, tacky and pale mucous membranes, and decreased skin turgor) were noted in both the control and blood collection groups. Throughout the study, all dogs exhibited normal behavior and appetite, and the clinical observations were not considered related to an adverse effect of the procedures.

**Body weight.** There were no statistically significant differences in body weight (Fig. 1). Throughout the study, the mean body weight of control dogs was  $10.87 \pm 1.35$  kg (range, 8.4 to 12.7 kg), and the mean body weight of the blood collection group was  $10.29 \pm 1.25$  kg (range, 8.2 to 12.5 kg). The control group lost an average of 1.15 kg whereas the blood collection group lost an average of 0.60 kg over the duration of the study.

**Hematological and serum chemistry features. (i) Blood morphol-**

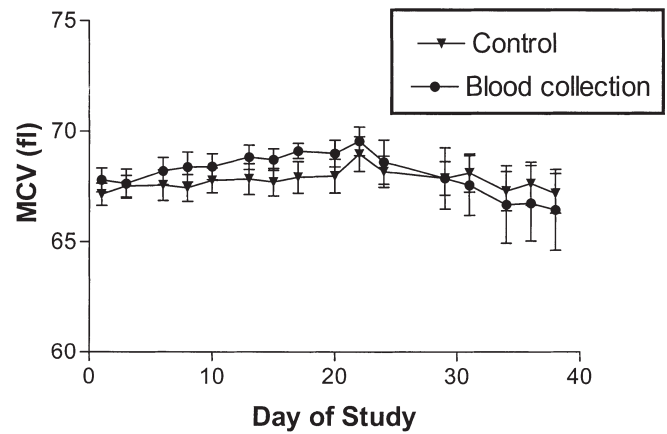


Figure 5. Mean cell volume (mean  $\pm$  standard error of the mean) over time.

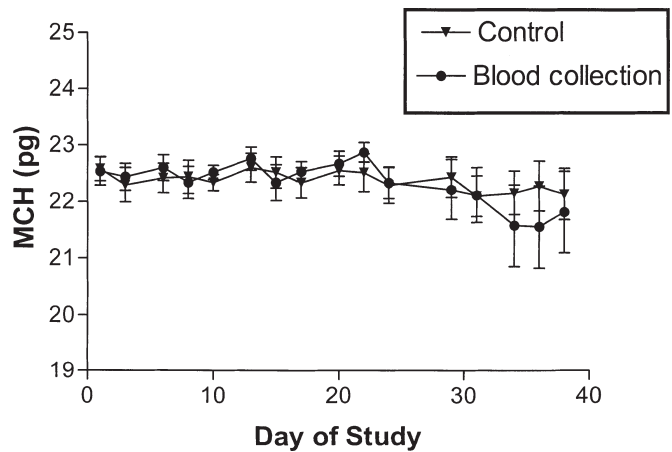


Figure 6. Mean cell hemoglobin (mean  $\pm$  standard error of the mean) over time.

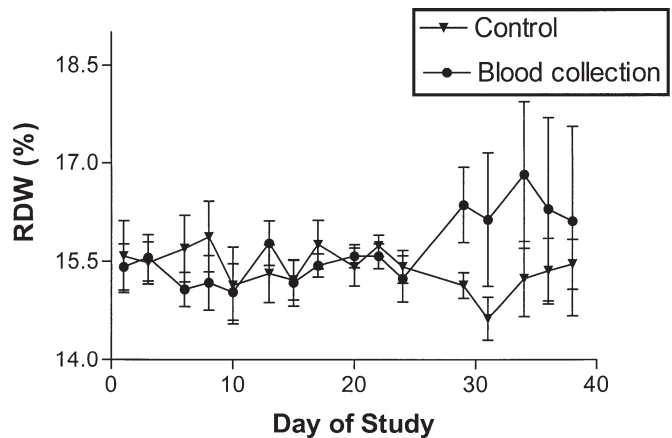


Figure 7. Red blood cell distribution width (mean  $\pm$  standard error of the mean) over time.

**ogy.** Blood morphology findings can be attributed to frequent blood collection, but there was not a difference in the findings in the control and blood collection groups. Anisocytosis was the most common finding in both groups. In the control group 87.5% of the dogs exhibited anisocytosis during the first 4 weeks of the study. In the blood collection group, anisocytosis was exhibited at a frequency of 60% during the first 4 weeks of the study. Polychromasia was seen at an equal frequency in control and blood collection dogs. Both groups also demonstrated Howell-Jolly bodies and clumped platelets; these changes appeared sporadically throughout the 6 weeks. Target cells were seen in both female groups on one occasion each. Hemoglobin crystals were found in one female beagle in the blood collection group during the final phase of the study. Hypochromasia

was found in a female dog in the blood collection group during the final 2 weeks of the study.

(ii) **Hematocrit.** Figure 2 illustrates the mean change in Hct during the study. The normal canine Hct in our laboratory is  $50.0\% \pm 3.3\%$ . The control group dogs' mean Hct was  $47.25\% \pm 3.13\%$  (range, 40.5% to 54.5%), whereas that of the blood collection group was  $41.63\% \pm 3.21\%$  (range, 34.3% to 50.0%). There were several days when the difference between the control and blood collection groups was significant: day 6 ( $P = 0.0016$ ), day 10 ( $P = 0.002$ ), day 13 ( $P = 0.0011$ ), day 17 ( $P = 0.0062$ ), day 20 ( $P = 0.0019$ ), day 22 ( $P = 0.0013$ ), day 24 ( $P = 0.0064$ ), and day 38 ( $P = 0.0078$ ).

(iii) **Total protein.** Figure 3 displays the changing mean total protein throughout the study. No statistically significant difference between the control and blood collection group was noted. The mean laboratory historical normal is  $5.6 \pm 0.20$  g/dl. The mean total protein in the control group throughout the study was  $5.47 \pm 0.20$  g/dl (range, 5.1 to 6.0 g/dl), whereas the blood collection group averaged  $5.39 \pm 0.22$  g/dl (range, 4.7 to 6.2 g/dl).

(iv) **Reticulocyte count.** There was no statistically significant difference in reticulocyte count between the treatment and control group throughout the course of the study (Fig. 4). Mean reticulocyte count in our laboratory for dogs is  $50,000/\mu\text{l} \pm 20,000/\mu\text{l}$ . The mean reticulocyte count for the control group was  $84,300 \pm 45,200/\mu\text{l}$  (range, 30,200 to 233,200/ $\mu\text{l}$ ), whereas that of the blood collection group was  $85,900 \pm 51,500/\mu\text{l}$  (range, 17,800 to 298,200/ $\mu\text{l}$ ). Both the control and blood collection groups had mild elevations in absolute reticulocyte counts.

(v) **Mean corpuscular volume.** There were no days when the control and blood collection groups had a statistically significant difference in MCV (Fig. 5). The mean in our laboratory for MCV is  $71.1 \pm 1.9$  fl. Dogs in the control group had a mean MCV of  $67.74 \pm 1.54$  fl (range, 64.2 to 72.0 fl), whereas that of the blood collection group was  $68.08 \pm 2.05$  fl (range, 60.7 to 71.2 fl).

(vi) **Mean corpuscular hemoglobin.** There were no days when the control and blood collection groups had a statistically significant difference in MCH (Fig. 6). The mean in our laboratory for MCH is  $23.4 \pm 0.8$  pg. Dogs in the control group had a mean of  $22.38 \pm 0.686$  pg (range, 20.5 to 23.6 pg), whereas that of the blood collection group was  $22.33 \pm 0.79$  pg (range, 19.1 to 23.7 pg).

(vii) **Red blood cell distribution width.** No statistically significant difference between the control and blood collection group was noted for RDW (Fig. 7). The mean RDW in our lab is  $15.6\% \pm 0.9\%$ . The mean value for the dogs in the control group was  $15.4\% \pm 0.87\%$  (range, 13.5% to 17.5%), whereas that of the blood collection group was  $15.7\% \pm 1.24\%$  (range, 13.7% to 21.7%). In the blood collection group, there was an increase in the standard error of the mean in RDW after day 28 of the study, but this finding was not statistically significant.

(viii) **All other hematological parameters.** All study dogs remained within laboratory normal limits for leukocyte count, differential leukocyte count, PT, APTT, and fibrinogen (data not presented).

## Discussion

Removal of as much as 15% of a dog's estimated blood volume during a 9-h period weekly for 4 weeks resulted in no serious side effects.

Important factors to assess in a dog suspected of being anemic are complete blood count with special attention to red blood cell morphology, indices, and reticulocyte counts (9-11). Evaluation of hematocrit, correlating with total protein for assessing blood loss and dehydration status, is also important (4, 5). Coagulation profiles were monitored to ensure that none of the dog's values were outside of the accepted historical normal range. Microcytosis was evaluated as an index of iron deficiency (12). Any abnormal hematological finding

on study dogs was correlated with daily observations for clinical signs to fully evaluate the animal's response to the blood collections.

The lack of any significant difference in the rate of weight loss in the blood collection group when compared with the control group suggests that removal of 15% of the blood volume does not cause ill effects on the animal's general health and well-being. The control and blood collection groups both lost weight, but blood collection preparation, basic collection techniques, and blood removal can stress an animal, regardless of the volume of blood removed (1, 2, 4, 13). The stress of repeated phlebotomy for 6 weeks probably accounts for the weight loss exhibited by all dogs in both groups. When the study started, animals were also limit-fed 300 grams a day, except for one female dog in the blood collection group who was observed to be in poor body condition; this dog's ration was increased to 350 grams of food a day on day 8 of the study. Because all dogs except one were limit-fed, weight loss was not unexpected with new stressors (handling and frequent blood collection) being experienced by the animals.

The most common finding on blood cell morphology was anisocytosis, which is a variation in red blood cell size sometimes indicative of red blood cell regeneration (9, 11). Both the control and blood collection groups demonstrated this variation. Anisocytosis has been described as a common, expected occurrence in normal dogs (11), but it is also consistent with a response to acute blood loss (9-11). Polychromasia, an increase in basophilic staining of erythrocytes, is normal in dogs, but it also increases during red blood cell expansion and regenerative anemia (9, 11). Howell-Jolly bodies are nuclear fragments left in the erythrocyte after the nucleus has been extruded (9, 11). Target cells are erythrocytes with a bright center surrounded by a paler concentric ring. All of these morphologic changes seen throughout the course of the study were exhibited equally in both groups. Hypochromasia, palely stained erythrocytes, is produced during states of iron deficiency (11), and hypochromatic erythrocytes were found on one day in a dog in the blood collection group, but lack of consistency and correlating clinical signs with this finding makes us conclude that this dog was not experiencing an iron-deficiency anemia.

The physiologic response to acute blood loss is regenerative anemia (9, 10). A low Hct causes the bone marrow to begin red blood cell production and to release reticulocytes into the peripheral circulation. The bone marrow needs sufficient time to respond, and maximum erythropoiesis usually takes place 4 to 5 days after an episode of hemorrhage (9, 10, 14). Shift or stress reticulocytes released in response to blood loss are macrocytic (10, 15, 16). In the present study, reticulocyte counts were closely monitored to ensure that they increased and that sufficient regeneration was occurring in the dogs between blood collections. Both groups showed an increase in reticulocyte count on days 3 and 6, but neither was more substantial than the other. The control group experienced mild reticulocytosis due to the stresses of handling and blood collection for clinical pathology 3 days a week for 6 consecutive weeks in a row. A normal absolute reticulocyte count range for canines is 0 to 60,000/ $\mu\text{l}$  (9). The highest reticulocyte count in our study was in a dog in the blood collection group (298,200/ $\mu\text{l}$ ) and reflects a response to an acute episode of blood loss. Because reticulocytes have basophilic stippling and are usually larger than normal red blood cells, the presence of reticulocytes will increase the erythrocyte indices.

In both groups of dogs, the mean MCV was less than our laboratory's mean, even though regeneration was occurring as evidenced by intermittent increased reticulocyte counts. The efficacy of monitoring MCV to assess the effects of removal of large quantities of blood has been questioned by other scientists (6). RDW is a calculated value which is provided by some laboratories and provides a measure of anisocytosis and is expressed as a percentage (17). Normal RDW for dogs is usually 16%. RDW is thought by some to be



a more accurate indicator of red blood cell regeneration than MCV, and this study has found this true. While MCV changed very little, RDW changed appropriately, especially in the blood collection group during the last 2 weeks of the study. RDW is a more sensitive indicator of blood loss than is MCV because fewer macrocytes are required to increase this value (9, 11). Malikides and colleagues found that removing 25% of the blood volume from horses changed the MCV values very little, whereas RDW increased 2 to 3 days after blood removal and stayed elevated for at least 42 days after blood removal (6).

Chronic blood loss would result in a period of regeneration followed by progressive deficiencies of iron and hemoglobin and the appearance of microcytic, hypochromic erythrocytes (9, 12, 18, 19). Three indices will change: MCV, MCH, and RDW all will decrease (12, 18). In our study, the blood collection dogs showed an increase in all three indices during the first 4 weeks of the study and remained within normal ranges throughout the study, indicating no evidence of microcytosis or iron deficiency anemia.

We also monitored Hct and total protein to assess dehydration status. Plasma protein is often decreased with acute hemorrhage because of the loss of plasma simultaneously with erythrocytes (5). The typical adult human fluid volume consists of 60% plasma and 40% blood cells. Although this volume can vary considerably between species, blood contains more plasma proteins than cells, so loss of blood can cause a similar loss of plasma proteins (20). Hemoconcentration, with an increase in total protein or albumin, would distinguish a dehydrated dog from a dog experiencing polycythemia. It is important to evaluate total protein and Hct along with clinical signs of dehydration. In the dogs in our blood collection group, clinical findings suggestive of dehydration could not be correlated with alterations in Hct and total protein.

None of our subjects showed severe clinical signs of shock or any other life-threatening condition from this blood collection regimen. This finding is consistent with similar results by Scipioni and colleagues, who evaluated removal of up to 40% of a rat's total blood volume without observing any clinical ill effects (3, 8). To our knowledge, our study is the first to evaluate the effects of taking 15% of the blood volume weekly from dogs. The group sizes were small, but the sampling frequency was large, giving our study statistical power by use of repeated measures. A useful follow-up would be a study that includes additional dogs and additional groups in which the percentage of blood volume removed was changed, e.g. 5%, 10%, 15%, 20%, and 25%. This inclusion of additional groups would help define the range of effects and pinpoint a time at which collection of large volumes of blood would affect the health of the dog and the science of the study. However, we conclude that, on the basis of our findings, collection of blood equaling approximately 15% of the blood volume weekly for 4 consecutive weeks can be accepted as a safe volume to remove from dogs. Individual dogs may have adverse reactions to large volume blood removal, and those animals that show subtle signs such as weakness, pallor, hypoactivity, or poor appetite, or severe signs of acute decompensation, shock, or dehydration should receive proper medical treatment as deemed necessary. Under the conditions described in the present study, this recommendation is compatible with maintaining the health and well-being of the dog and can be acceptable in laboratory situations when scientifically justified.

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