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Comparison of Serial Blood Collection by Facial Vein and Retrobulbar Methods in C57BL/6 Mice

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Many biomedical research protocols for mouse models involve serial blood collection and analysis. Two common techniques for serial blood collection in this species are the retrobulbar (RB, also called retroorbital) and facial vein (FV) methods. However, previous studies comparing these methods typically evaluated collection at a maximum of 2 time points. Here we compared hematologic values, adverse clinical effects, and histopathologic lesions in mice bled either once or serially (6 times) by using the FV or RB method. Mice (*n* = 48) were divided into 4 groups: single FV, single RB, serial FV and serial RB. Mice in the single-collection groups underwent a single blood collection by the indicated method, whereas those in the serial-collection groups were sampled once weekly for 6 consecutive weeks. All animals were euthanized and necropsied 2 wk after their last blood collection. Compared with all other groups, the serial FV group experienced more serious clinical adverse events, including 33% mortality, convulsions, head tilt, and hemorrhage from the ear canal and nares. In addition, mice in the FV groups had a significantly greater acute body weight loss compared with mice in the RB groups. Histologically, mice in both serial-collection groups had an increased incidence of tissue lesions compared with their respective single-collection groups. Importantly, only mice in the serial FV group had life-threatening histopathologic lesions, including cerebral hemorrhage or ischemia. Given these data, we conclude that serial blood collection in mice causes increased incidence of tissue damage compared with single sampling, and serial blood collection by the FV method causes substantial morbidity and mortality compared with the RB method.

Abbreviations: BW, body weight; FV, facial vein; RB, retrobulbar; TBV, total blood volume

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Laboratory mice are the most commonly used animals for in vivo biomedical research, and many of these investigations require blood collection for analysis. However, blood sampling in mice can present a challenge due to their small size, especially when the study requires large volumes of blood collected at multiple time points. Blood sampling techniques include retrobulbar (RB; commonly referred to as retroorbital), facial vein (FV), tail vein, lateral saphenous vein, sublingual vein, submental vein, tail-tip amputation, and jugular vein.[11,12,14,19,26] For each study, the most appropriate blood sampling technique depends on the frequency and volume of sample required, required restraint method, skills and abilities of the operator, potential impact of collection on data collected, and the health and welfare of the animal.[14,20,26]

The 2 most commonly referenced techniques for obtaining large volumes of blood for serial blood collection are the FV and RB methods.[22] In recent years, the biomedical research community has increased restrictions on the RB blood collection method due to concerns of tissue damage.[5,9,12,13,22] The NIH now recommends the use of anesthesia or topical anesthetic when using the RB procedure.[20] In fact, many IACUC, including our own, currently do not allow RB blood collection in rodents without anesthesia unless given adequate scientific justification. Since this policy was implemented, our institution has seen an increased frequency of and even recommendation for using the FV method for blood sampling in mice. Conversely, several recent studies report that the FV method is more stressful when compared with other phlebotomy techniques.[19,25,26] Adverse effects associated with the FV method include bleeding from the ear canal,[9] unsteady gait after blood collection,[4] and excessive hemorrhage in coagulation-compromised mice.[16]

Both RB and FV blood collection methods have the potential to cause serious tissue trauma due to the anatomic location of the collection site. The FV method collection site is in a large muscle group close to the facial nerve, mandible, temporomandibular joint, and auditory canals. Any deviation in the puncture site has the potential to result in adverse bleeding events, such as hemorrhage from the ear canal, hemorrhage from the nares, head tilt, and even laceration of facial muscles and nerves.[8,9] The RB method collection site is deep to the ocular globe, which encompasses many sensitive soft-tissue structures, such as the conjunctiva, Harderian gland, extraocular muscles, and the globe. Adverse events of RB bleeding include lesions in the Harderian gland and adnexa, ocular discharge, corneal lesions, and severe ocular damage.[10,12,27] Because both methods are near sensitive tissues and because the phlebotomist cannot visualize the vessels, the training and experience of the phlebotomist become important for successful blood collection and the ultimate the welfare of the animals being bled. Training and experience in the RB method has been shown to have significant effects on the animal,[10,27] and as little as 1 h of hands-on practical training can be sufficient to minimize clinically adverse effects from phlebotomy.[10]

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Although several studies have compared the effects of FV or RB blood collection, most evaluated only 1 or 2 blood samples[9,15,16,23,25,26] and did not address the potential repeated tissue trauma caused by serial blood collection. One recent study examined the effects of serial RB blood collection alone on animal health but did not include histopathologic analysis of the RB region for evidence of tissue damage.[21] Therefore, to inform institutional policy, we sought to compare the effects of single or serial blood collection by using either the FV or RB blood collection techniques. Effects evaluated included CBC, clinical blood chemistry, histopathologic evidence of tissue trauma, and acute change in body weight (BW) and clinical observations of adverse reactions to blood collection. In light of personal experience and previous studies indicating increased animal stress during FV blood collection, we hypothesized that the FV method would be a less desirable method of serial blood collection according to markers of animal health and welfare. In addition, we hypothesized that, due to the nature of repeated trauma, serial blood collection would cause significantly more tissue damage at the collection site than single blood collection.

Materials and Methods

Animals, husbandry, and welfare. Female C57BL/6NCrl mice (age, 12 to 14-wk) were obtained from Charles River (Wilmington, MA) and were maintained in facilities in which sentinel mice tested negative for the following agents: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Theiler murine encephalomyelitis virus, reovirus, rotavirus, mouse adenovirus, lymphocytic choriomeningitis virus, ectromelia virus, K virus, mouse cytomegalovirus, mouse norovirus, hantavirus, lactate dehydrogenase elevating virus, *Mycoplasma pulmonis*, and *Helicobacter* spp. In addition, sentinel mice were free from helminths and external parasites. Mice were housed 4 to a cage and kept on a daily 12:12-h light:dark cycle in a temperature-controlled room in an AAALAC-accredited vivarium. Animals were provided with standard rodent chow (8640, Teklad 22/5 rodent diet, Envigo, Somerset, NJ) and water without restriction. Mice were acclimated to their new environment inside the vivarium for 7 d prior to the start of the study and were ear punched for identification purposes at 3 d before. All animal care was performed in accordance with the standards in the *Guide for the Care and Use of Laboratory Animals.* The IACUC of the University of California San Diego approved all procedures performed. Mice were observed daily by animal husbandry technicians for any signs of lethargy, scruffy coat, or other signs of illness. If any animal was noted to be moribund, veterinary staff was immediately notified and that animal was euthanized.

Experimental design. Cages were randomly assigned to 4 groups (n = 12 each) for blood collection: single RB, serial RB, single FV, and serial FV. The single-collection groups were bled once (week 1) according to the assigned technique and then euthanized and necropsied 2 wk later (week 3). The serial-collection groups were bled once weekly for 6 wk, starting at week 1, and then euthanized and necropsied 2 wk after the last blood collection (week 8). For all time points, all mice were bled on the same day in the same room by the same person (AS for FV, JF for RB). Both persons had significant prior experience (more than 10 mo) in the assigned technique. All blood collections were performed on the animal's right side. Approximately 250 µL (equal to 18% to 24% of the total blood volume [TBV] according to the starting weights of the heaviest and lightest mice) was collected from each mouse at each time point; this volume is within the range of 'safe' amounts to collect weekly from female C57BL/6 mice.[21]

RB technique. Animals were anesthetized by using inhaled isoflurane, and adequate depth of anesthesia was confirmed by lack of pedal reflex (toe pinch). Once an adequate plane of anesthesia was achieved, blood was collected by using a 75-µL heparinized microhematocrit tube (Fisherbrand, Fisher Scientific, Pittsburgh, PA) as previously described.[14] Briefly, mice were placed in left lateral recumbency, and the end of a capillary tube was inserted into the lateral canthus and under the right globe. Blood flowed from the microhematocrit tube directly into an EDTA microcollection tube (Microtainer, Becton Dickenson, Franklin Lakes, NJ). After the microhematocrit tube was removed, clean dry gauze was applied over the eye until hemostasis was achieved. Mice were then placed in a recovery cage for the observational period.

FV technique. Mice were restrained manually by scruffing the skin of the dorsal neck. A 5-mm lancet (Goldenrod Animal Lancet, Medipoint, Mineola, NY) was used to puncture the facial vein on the right side of the face using landmarks previously described,[11] approximately 0.5-cm dorsal and caudal to facial scent gland. Blood was allowed to drip freely into an EDTA microcollection tube (Microtainer, Becton Dickenson). Hemostasis was achieved by using clean, dry gauze applied directly to the access site. Mice were held by the scruff until hemostasis was achieved and then placed into a recovery cage for the observational period.

BW measurement and other calculations. Immediately before and after blood collection, all mice were weighed on a digital scale (model LT620, Acculab, Newtown, PA) to the nearest 0.01 g. To minimize variability in BW measurements, the scale was set to the 'animal weighing' mode specifically designed for weighing live animals. Animals were weighed in a tared small plastic beaker centered on the pan of the scale, and the beaker was cleaned between animals so that any urine or feces that occurred during the weighing process was included. When a mouse was overly excited or active and did not rest at the bottom of the beaker during weight measurement, the animal was removed from the beaker and the measurement repeated. Acute BW change was calculated by subtracting the preblood collection weight from the postblood collection weight. This value was used to estimate the absolute amount of blood lost during blood collection. Percentage BW change in serial groups over the course of the entire study was calculated by taking preblood collection weights from week 1, subtracting it from the prenecropsy weight in week 8, dividing by the week 1 weight, and multiplying by 100%. The BW change from week 1 to week 3 in all groups was calculated by subtracting the preblood collection weight in week 1 from the preblood collection weight in week 3, dividing by the initial weight, and then multiplying by 100%. The percentage of TBV was calculated by multiplying the BW by 0.06[21] and then multiplying by 100%.

Clinical observations. The same observer (TH) monitored mice continuously for 1 h after blood collection to evaluate recovery and to record clinical signs of adverse effects, before the mice were returned to their home cages in the housing room. Adverse effects were recorded according to individual mouse and included hemorrhage from nares, hemorrhage from the ear canal, corneal opacity, periorbital tissue prolapse, head tilt, circling, convulsions, and reduced activity. Reduced activity was defined as any mice that did not actively move within 15 min after blood collection. Any mice that appeared moribund were euthanized and necropsied.

Necropsy and histopathology. Mice were euthanized by using carbon dioxide inhalation in accordance with the AVMA *Guidelines on Euthanasia*.[2] Mice were placed in a designated euthanasia chamber of known volume, and CO_2 was flowed into the chamber at a maximal rate of 30% of the chamber volume per minute by using a gas outflow regulator. The euthanasia chambers were inverted after each cage of mice was euthanized to ensure that the residual CO_2 was removed prior to the next cage being placed into the chamber. Once respiration ceased, mice underwent cardiocentesis for clinical biochemistry analysis, and representative tissues were collected for histopathology. The entire head, internal viscera, sternum and ribs, and right leg were preserved in 10% buffered formalin. Bony tissues (head, leg) were decalcified (Cal-Ex, ThermoFisher Scientific, Waltham, MA) for 43 to 48 h prior to tissue trimming. Sections of liver, bone marrow (either in sternum, rib, or femur) and at least 2 transverse (coronal) sections of the head were processed for paraffin embedding, and then stained routinely with hematoxylin and eosin. Sections of the head were taken at the level of the internal ear canals and at the level of the midorbit (Figure 1); additional sections were obtained when gross pathology was noted during tissue trimming. Serial sections (4 at 100 µm apart) of the head of one animal from each serial group (FV and RB) were evaluated also. Stained sections were examined for the following parameters: presence and severity of soft tissue hemorrhage, hemosiderosis, granulation tissue, fibrosis, edema, necrosis, and inflammation; auditory canal lesions; ocular lesions; presence of all 3 bone marrow lineages; and degree of hepatic extramedullary hematopoiesis. For animals that were euthanized or died prior to their cohort endpoint, additional tissues were examined, including heart, lung, kidney with adrenal glands, spleen, intestines with the pancreas, and reproductive tract. The pathologist was blinded to the cohorts but did participate in the tissue collection and trimming.

Hematology analyses. Hematology was performed on an automated analyzer (HemaVet HV950, Drew Scientific, Miami Lakes, FL) by using mouse-specific settings. All hematology samples were run the same day as collection. Parameters determined included WBC count and differential, RBC count, Hgb, Hct, MCV, MCH, MCHC, RDW, platelet count, and MPV. Criteria for anemia were determined according to a calculation described previously.[21] Briefly, baseline Hgb values for each serial group were determined by calculating the mean Hgb concentrations from the week 1 blood collection. Anemia then was defined as a Hgb concentration lower than 2 SD below the baseline for each group. When anemia was noted in a sample, blood smears stained with new methylene blue were examined to obtain a manual reticulocyte count. The normal range of hematology values for female C57BL/6NCrl mice in the same age range as study mice was obtained from the Charles River website.[3]

Clinical chemistry analyses. Blood for clinical biochemistries was collected by cardiocentesis after euthanasia by CO_2 inhalation. When insufficient blood volume was collected by using cardiocentesis, blood in the abdominal cavity was collected by using a syringe. Whole blood was transferred into Li–heparin plasma separator microcollection tubes (Microtainer, Becton Dickenson), and then centrifuged. Plasma was transferred into clean microcentrifuge tubes (Eppendorf, Hauppauge, NY) and frozen at –80 °C until analysis. An automated analyzer (Vet Scan VS2, Abaxis, Union City, CA) was used with rotors that included reagents for albumin, ALP, ALT, amylase, BUN, calcium, creatinine, globulin (calculated), glucose, phosphorus, potassium, sodium, total bilirubin, and total protein. A normal range of clinical chemistry values for female C57BL/6NCrl mice of the same age range as study mice was obtained from the Charles River website.[3]

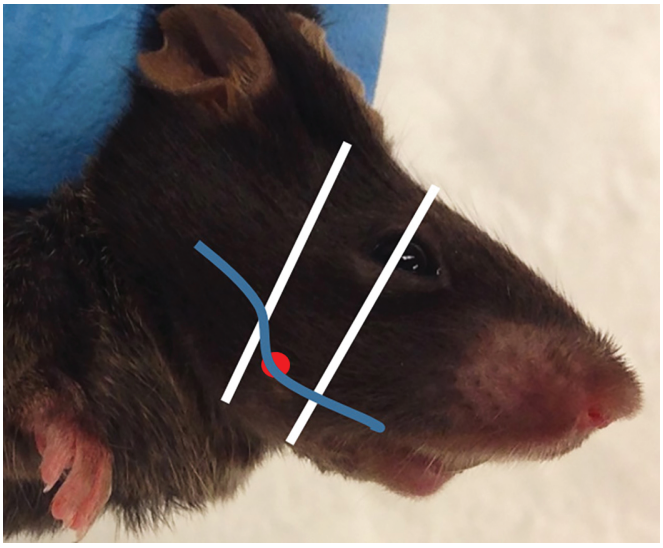

Figure 1. A representative restrained mouse with markings to indicate approximate location of sections taken for histology (white lines), location of the facial vein (blue line), and region of blood collection (red dot).

Statistical analysis. All data were analyzed in JMP (version 2009, SAS Institute, Cary, NC). For hematology and acute BW change values, a linear mixed-effects model was used with the group (FV or RB), week, and their interaction as fixed effects and mouse ID as random effects. Posthoc Tukey Honestly Significant Difference or Student *t* tests were performed to identify significant interactions. Prior to running the linear mixed-effects model analysis, values from week 1 were analyzed by using a paired *t* test to determine whether single and serial values for each group (FV and RB) differed significantly. When they were not, all values from the single-collection groups were added to week 1 of their respective serial-collection group prior to linear mixed-effects model analysis. When values differed statistically between single and serial frequency in the same group, linear mixed-effects model analysis was run using only values from the serial groups. Clinical chemistry values were analyzed by using 2-way ANOVA with posthoc Tukey adjustments to determine the significance of any interactions. Differences between the serial FV and serial RB group in percentage BW change from week 1 to week 8 were calculated by using unpaired samples *t* tests. One-way ANOVA with group as a factor was used to calculate differences in percentage BW change from week 1 to week 3 across all groups. Any nonnormally distributed data were log-transformed to meet the assumptions of the statistical test. Data were transformed rather than evaluated by using nonparametric tests to keep analyses consistent across variables.

Results

Clinical adverse effects. During the 1-h postcollection observation period, the serial FV group had the most mice with overt clinical adverse effects, followed by serial and single RB groups, and lastly the single FV group, which had no overt clinical adverse effects (Table 1).

Mortality. Of the 12 mice in the serial FV group, 4 (33%) did not survive to the study endpoint. Mouse 21L was found dead on day 14 without any observed clinical signs, and mouse 21RL was found dead on day 27 after a history of postcollection convulsions on day 21. On day 13, mouse 25R acutely

Table 1. Clinical adverse effects observed during the 1-h postcollection period

	Single RB	Single FV	Serial RB	Serial FV
Reduced activity	0	0	0	2
Corneal opacity	2	0	5[a]	1[c]
Periocular tissue prolapse	2	0	1[a]	0
Hemorrhage, nares	0	0	0	1
Hemorrhage, ear canal	0	0	0	2[b]
Circling	0	0	0	1[b]
Head tilt	0	0	0	2[b,c]
Convulsions	0	0	0	3[b]

Data are presented as the number of mice affected per adverse effect.
[a,b,c]Mice with multiple adverse effects.

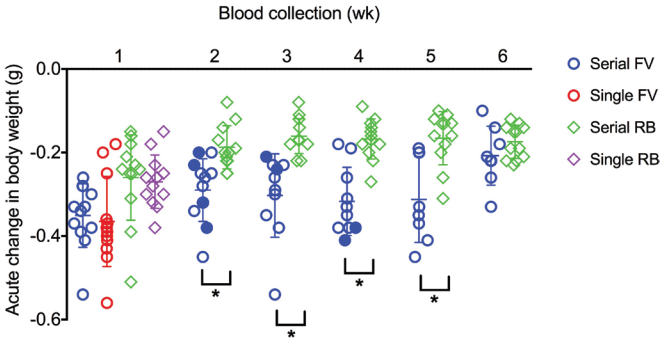

Figure 2. Acute change in mean body weight (bar, 1 SD) in all groups over time. Solid blue circles represent mice that were euthanized or died before study completion. *, Time points at which the acute change in body weight was significantly ($P < 0.05$) greater in the FV group than the RB group.

moribund without a history of adverse events and immediately euthanized. Mouse 21N was euthanized on day 22, when it was found to be moribund after a history of several adverse events, including hemorrhage from the ear canal, head tilt, and postcollection convulsions.

BW. Analysis of acute BW change by using a linear mixed-effects model revealed significant ($P < 0.0001$) effects of group and week. Specifically, weight loss during weeks 2 through 5 was greater in the FV group than the RB group. The acute BW loss that occurred during week 1 was significantly ($P < 0.05$) greater than in all other weeks (Figure 2). Percentage BW change throughout the entire study (weeks 1 through 8) did not differ significantly between the serial FV (mean ± 1 SD, 15.2% ± 6.3%) and serial RB (18.0% ± 12.1%) groups ($t_{18} = 0.58$, $P > 0.1$). Four mice in the serial FV group were excluded from this analysis due to premature death. No significant differences emerged when we compared percentage BW change from week 1 to week 3 among all groups (serial FV, 1.5% ± 0.5%; serial RB, 0.9% ± 0.2%; single FV, 0.2% ± 4.0%; single RB, 2.1% ± 3.6%; $F_{3,45} = 1.08$, $P > 0.1$). Two mice in the serial FV group were excluded from this analysis due to premature death.

Necropsy and histopathology. Histologic lesions for each group are summarized by lesion type in Figure 3. When present, lesions were unilateral, occurring on the animal's right side. In the single-collection FV group, one animal had mild, unilateral, neutrophilic, and histiocytic steatitis of the adipose deep to the facial muscles with intralesional hair shafts, consistent with foreign body (hair shaft)-induced inflammation (Figure 4 A). In the serial-collection FV group, the cause of morbidity or mortality was identified for only 2 of the 4 mice. In particular, mouse 21RL had postcollection convulsions on day 21 and was found

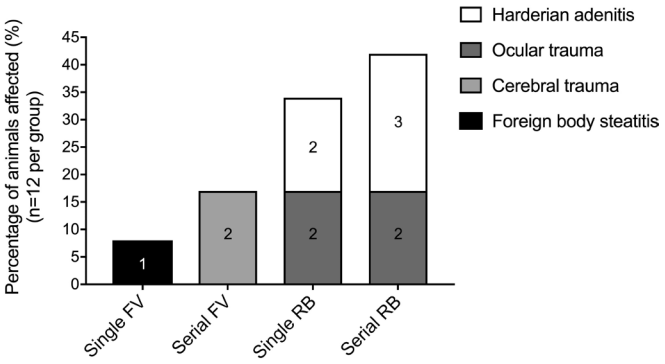

Figure 3. Percentage of mice with various histologic lesions in each blood collection group (12 mice per group). The number inside the bar represents the absolute number of animals with the described lesions. Total percentages of affected animals by group: single FV, 8%; serial FV, 17%; single RB, 34%; and serial RB, 42%.

dead on day 27; this animal had unilateral midbrain hemorrhage (Figure 4 B) and vascular changes including hyalinization and thickening of vessel walls and partial occlusion of vascular lumina (Figure 4 C), with increased glial cells and occasional leukocytes. Mouse 21N demonstrated hemorrhage, convulsions, and a persistent head tilt from day 7 until euthanasia on day 22 and had unilateral encephalomalacia of thalamic parenchyma (Figure 4 D), characterized by tissue rarefaction, increased glial cells and gitter cells, and dilated myelin sheaths with swollen axons (spheroids; Figure 4 E).

Mice in both RB groups had ocular-associated lesions (Figure 4 F), which were similar between the single and serial RB groups. In the single RB group, 2 animals had either mild or severe Harderian gland adenitis accompanied by disorganization and atrophy of ocular globe contents, consistent with phthisis bulbi. Three mice in the serial RB group had mild to moderate Harderian gland adenitis, and 2 of these 3 animals had accompanying phthisis bulbi. Step sections performed on one of these 2 animals showed glandular inflammation present in every section (spanning 400 µm in depth). Mild adenitis (Figure 4 G) was characterized by small aggregates of lymphocytes and plasma cells, whereas more severe (Figure 4 H) adenitis was characterized by dense populations of mixed leukocytes (predominantly neutrophils and macrophages) that obscured normal glandular epithelia and were admixed with fibroblasts, small regions of glandular necrosis, and glandular ectasia. Likewise, affected ocular globes in both groups (Figure 4 I) were characterized by generalized atrophy, intense fibrosis, and frequent disorganization to loss of intraocular tissue layers. Bone marrow and liver sections were within normal limits in all mice.

Hematology. Statistically significant differences in hematology values between serial FV and serial RB groups are summarized in Figures 5 and 6. Total WBC and lymphocyte counts were significantly ($P < 0.0001$) lower for week 6 compared with all other weeks (Figure 5 A and B). Neutrophil counts showed significant interaction between group and week (Figure 5 C), were greater ($P = 0.0033$) in FV than RB groups, and were significantly ($P < 0.0001$) lower in week 6 compared with all other weeks. In addition, mean neutrophils in week 4 were significantly ($P = 0.007$) increased in the serial FV group. Monocyte counts showed significant interactions of both group and week. The RB groups had significantly greater monocyte counts than FV groups ($P = 0.0289$). Statistically significant differences ($P < 0.05$) were revealed after comparing data for the following pairs of weeks (the time point with the greater monocyte count is listed first): weeks 4 and 1, 4 and 2, 4 and 6, 3 and 1, and 5

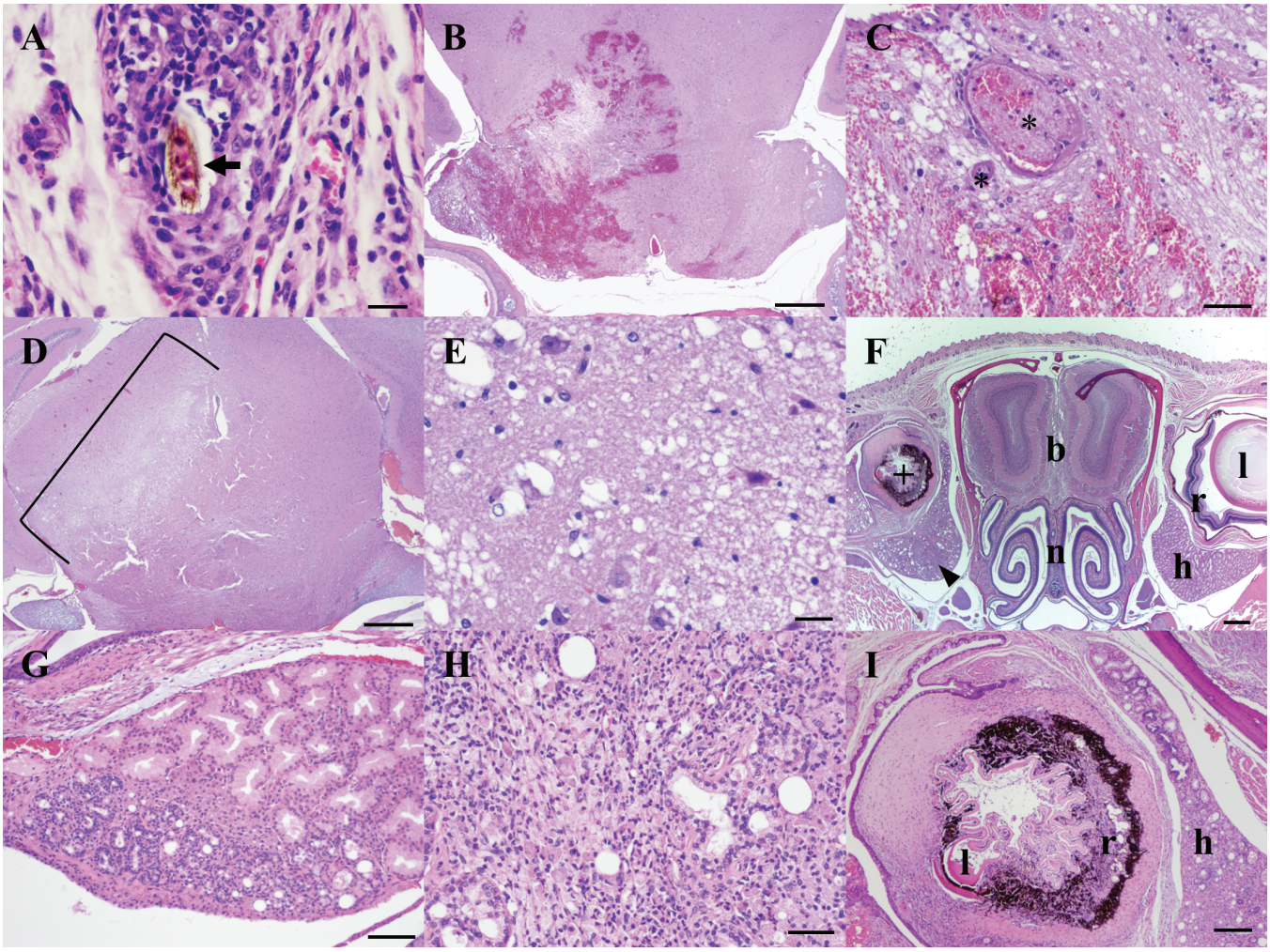

Figure 4. Representative histologic lesions from blood collection groups. (A) Single FV group, foreign-body steatitis; bar, 50 µm. Leukocytes surround a hair shaft (arrow). (B) Serial FV group, right midbrain hemorrhage; bar, 500 µm. This mouse was found dead a week after having a convulsion. (C) Serial FV group, vessels in areas of midbrain hemorrhage; bar, 50 µm. Vessel walls are expanded by hyaline material, and vascular lumina (*) are partially thrombosed. (D) Serial FV group, right thalamic encephalomalacia; bar, 500 µm. The unilateral tissue pallor (bracket) indicates parenchymal loss. (E) Serial FV group, region of encephalomalacia; bar, 20 µm. Increased inflammatory infiltrates and spheroid formation, suggestive of ischemic insult, are present. (F). Single RB group, Harderian gland adenitis and phthisis bulbi; bar, 500 µm. Compared with the contralateral eye, the affected Harderian gland (arrowhead) is densely cellular, and the affected globe (+) is shrunken. b, Forebrain; h, Harderian gland; l, lens; n, nasal septum; r, retina. (G) Single RB group, mild Harderian gland adenitis; bar, 100 µm. Small aggregates of lymphocytes are present in regions of glandular atrophy and ductal ectasia. (H) Single RB group, severe Harderian gland adenitis; bar, 50 µm. Dense leukocyte infiltrates obliterate glandular epithelia, and marked ductal ectasia and regional fibrosis are present. (I) Serial RB group, phthisis bulbi; bar, 200 µm. A disorganized and shrunken ocular globe, consistent with phthisis bulbi, shows marked, generalized fibrosis. Also present are a remnant lens (l), disorganized and fibrosed retina (r), and inflamed Harderian gland (h). Hematoxylin and eosin stain; images adjusted for brightness and contrast.

and 2. RBC counts were significantly ($P < 0.0001$) greater in the FV groups compared with RB groups (Figure 6 A). Specifically, RBC values during week 1 were greater ($P = 0.0016$) in the FV groups compared with the RB groups. Week 1 platelet counts differed ($P = 0.0151$) between single and serial FV groups (Figure 6 B); however, platelet counts did not differ between the serial groups over time.

According to Hgb values, only 2 mice (1 each from the serial FV and RB groups) were considered anemic during week 2 (Figure 7). The reticulocyte counts from these animals ranged from 14% to 65% (data not shown), suggesting a regenerative process. All hemoglobin values in both mice had recovered by the next sampling time point (week 3), and both mice remained nonanemic for the remainder of the study.

Clinical chemistry. Statistically significant differences in clinical chemistry values between single and serial groups for each of 2 blood collection methods are summarized in Figure 8. No significant differences emerged when we compared serial FV compared with serial RB groups. Chemistry values were significantly increased in both serial-collection groups compared with their respective single-collection groups for albumin ($P < 0.001$), total protein ($P = 0.03$) and BUN ($P < 0.0001$). ALP was decreased ($P < 0.001$) in both serial groups compared with their respective single groups. ALT ($P = 0.035$) was increased in the serial RB group relative to the single RB group. Many samples for clinical chemistry showed marked hemolysis. Creatinine was not included in the statistical analysis because readings for most samples were less than 0.2 mg/dL. ALT values ranged widely (30 to 1633 U/L), and the majority (71%) of samples were more than 1 SD higher or lower than normal. All other clinical chemistry values did not fall significantly outside the normal ranges.

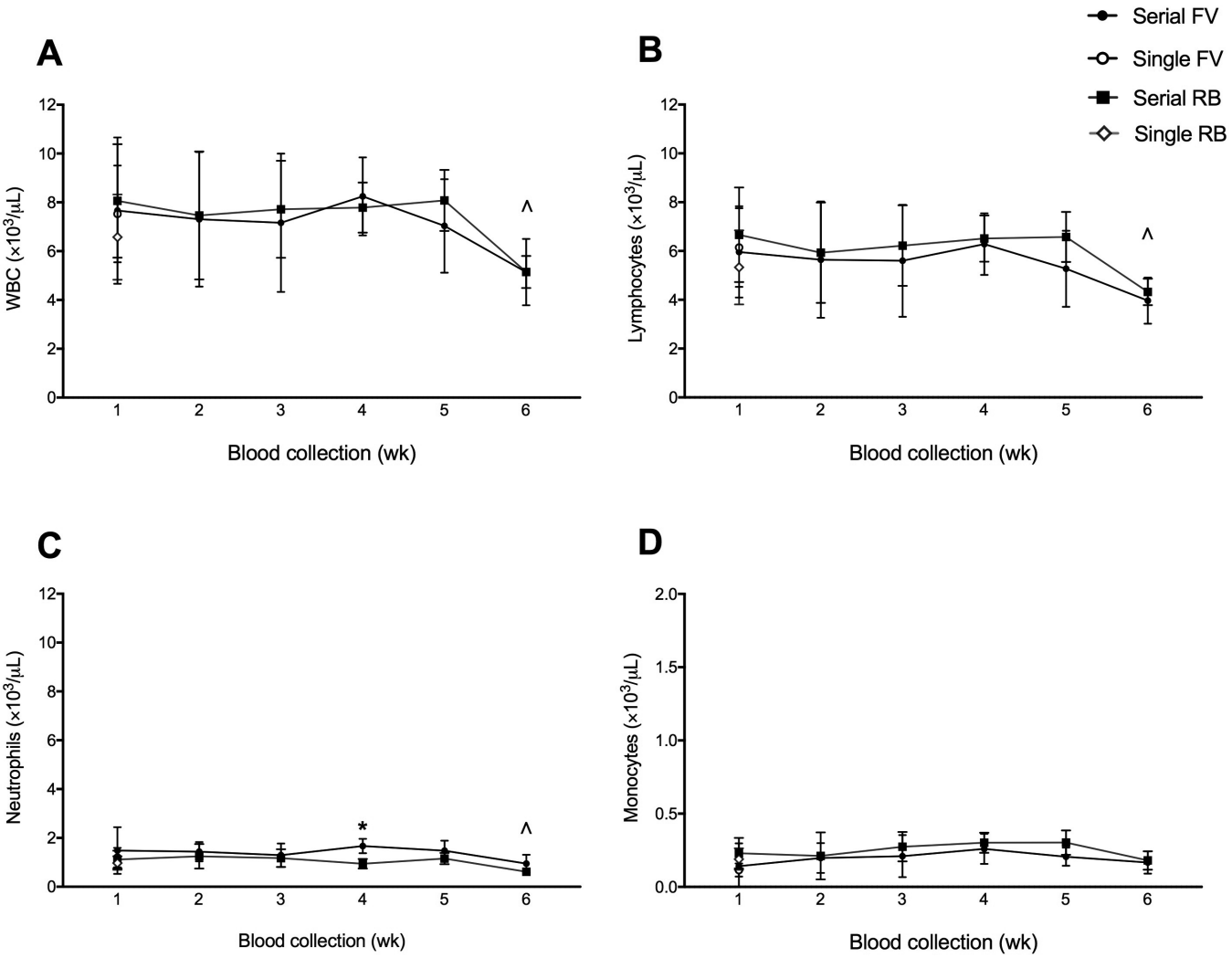

Figure 5. (A) WBC, (B) lymphocyte, (C) neutrophil, and (D) monocytes counts (mean ± 1 SD) for each group over time. *, Significant ($P < 0.05$) difference between FV and RB groups during the indicated week; ^, significant ($P < 0.05$) difference between time points for a given group.

Discussion

Our current data support the hypothesis that serial blood collection from mice by using the FV method had more adverse animal health outcomes than the RB method. Importantly, the serial FV group had the most severe clinical adverse events of all the groups and a troublingly high 33% mortality rate (4 animals). No other group experienced mortalities, suggesting that serial blood collection by using the FV method has an increased potential for trauma to the animal.

Although both RB and FV methods can cause blood loss and tissue damage, an alarming 33% of animals (4 total) in the serial FV group died or were euthanized in the present study despite the extensive experience of the phlebotomists. Two of the mice that died (animals 21N and 21RL) had histopathologic brain lesions suggestive of nonspecific parenchymal or vascular trauma and ischemia. These lesions are likely related to the clinical signs observed prior to death. In particular, immediately after blood collection, mouse 21N exhibited head tilt and hemorrhage from the ear canal, which results when the vein is pierced too far dorsally along the jaw.[9] In addition, both of these 2 animals had postcollection convulsions, which potentially were due to trauma, stress, or a combination of both. Another study showed similar clinical signs, namely seizures, in mice after the induction of ischemia and found that these seizures were tightly correlated with mortality.[6] Interestingly, a third mouse (in the serial FV group) had convulsions but lacked corresponding histopathologic brain lesions. In this particular case, transient damage caused on the day of the convulsive episode (week 3) might have resolved prior to tissue collection at necropsy (week 8).

Although neither of the other 2 cases of mortality in the FV serial blood collection group demonstrated specific histopathologic lesions, potential etiologies include anemia, acute blood loss, and stress. In this study, anemia was considered an unlikely cause of death because none of these mice were anemic according to hematologic values collected prior to death or at euthanasia. In contrast, anemic animals (present in both the serial FV and serial RB groups) recovered within 1 wk and survived to the study endpoint. Acute blood loss is another possible etiology for mortality events in mice; however, the data from the present study do not support this hypothesis. Mice that died had a wide range of acute BW change values (Figure 2) instead of an increase in acute BW change that correlated with mortality. However, it is interesting to note that the general trend for acute BW change was increased in animals bled by using the FV method compared with the RB method. This pattern may be indicative of a clinically relevant difference between the 2 blood collection methods. However, we acknowledge that the

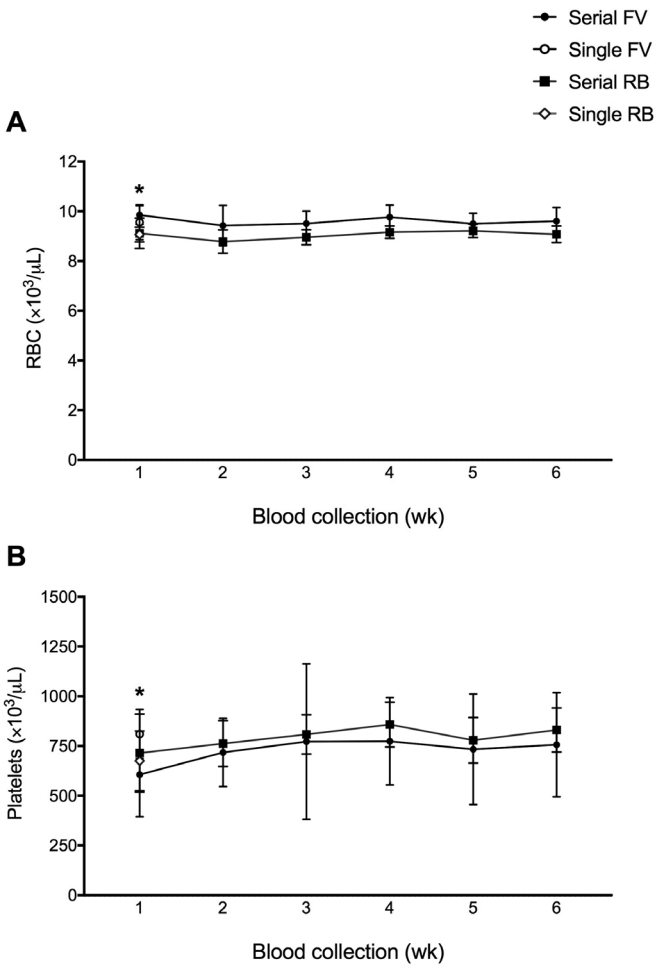

Figure 6. (A) RBC and (B) platelet counts (mean ± 1 SD) for each group over time. *, Significant ($P < 0.05$) difference between groups at the specified week.

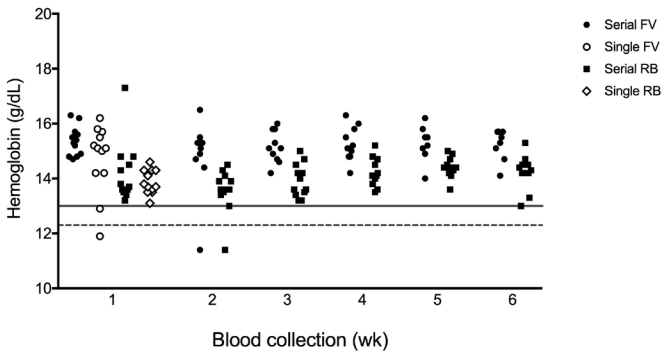

Figure 7. Dot-plot of individual Hgb values by group over time. The solid gray line represents the lower limit for mice in the serial-collection FV group to be considered anemic. The dotted line represents the lower limit for animals in the serial RB group.

use of acute BW change as a surrogate for measuring acute blood loss presents various challenges and limitations, because it does not account for potential internal hemorrhage and because a portion of the calculated acute BW change could be due to other losses, such as defecation, urination, or movement of the animal. Although efforts were taken to minimize variability during BW measurements, as described in the Materials and Methods section, these potential losses cannot be completely controlled. We considered alternative methods for measuring blood loss, such as direct collection into capillary tubes[10,21,26] and measuring volumes in collection tubes,[9,23] but these methods have their own limitations, such as the inability to account for blood not ultimately captured in the collection device (that is, internal hemorrhage and blood soaked into the gauze during hemostasis). Ultimately, we used acute BW measurement to quantify the total external blood loss during blood collection. By using this method, the results from this study suggest that mice in the FV groups may have lost more blood during collection than did those in the RB groups. It is important to keep in mind that, although it appears that mice in the serial FV group lost more weight acutely at the time of blood collection, the percentage weight change throughout the study (weeks 1 through 8) did not differ significantly between the serial FV and serial RB groups. In fact, all mice gained weight over the course of the study.

The biggest difference between animal handling in the RB and FV groups was the use of anesthesia for the RB groups as recommended by updated guidelines for humane blood collection.[20,22] However, the contribution of anesthesia (or the lack thereof) to the mortality rate in the serial FV group is difficult to ascertain. Although anesthesia alone can cause increased stress in mice,[24] FV blood collection without anesthesia has been shown to be more stressful than FV blood collection with anesthesia.[13] By measuring parameters such as corticosterone, other studies have shown that the FV method is more stressful than the RB method,[25,26] and mice lost more weight when they underwent FV blood collection.[25] Because these previous studies did not use anesthesia for either FV or RB groups, we cannot directly correlate the previous and our current study.

Histologically, blood collection in both the serial FV and RB groups resulted in an increased incidence of tissue damage as compared with their respective single-collection groups, as to be expected with repeated tissue trauma. Mice in the single and serial RB groups exhibited similar histologic trauma to the Harderian glands and globe, and these current results are consistent with previous findings.[12,25] Multiple studies have cited tissue trauma as an argument against using the RB blood collection method, especially for serial application.[7,9,12,13] However, the change in the number of incidences between the serial and single RB groups was surprisingly small, with serial collection resulting in only 1 more animal with histologic lesions. Therefore, our findings suggest that serial application of the RB method does not dramatically increase the risk for tissue damage. In fact, the most severe histopathologic lesions pertinent to overall animal wellbeing occurred in the serial FV group, in which 16% of animals had evidence of cerebral hemorrhage and ischemia. Although outside of the scope of our current study, previous work showed that using a needle rather than a lancet to access the facial vein resulted in larger (although not statistically significant) hematomas seen during necropsy.[9] However, because no histopathology was performed in the previous study, whether using a lancet compared with a needle in single compared with serial FV collection might lead to different amounts of tissue damage is unclear. In the present study, both RB groups had a greater absolute percentage of animals with histopathologic lesions than did both FV groups, but we consider that the types of lesions in the RB groups were less severe relative to overall animal wellbeing (that is, Harderian adenitis and ocular trauma are less life-threatening than cerebral hemorrhage). These histopathologic data correlate with the increased morbidity and mortality in the serial FV group as compared with all other blood collection groups.

Previous studies have shown that the site of blood collection can affect blood analysis, such as CBC values.[15,23] In the present

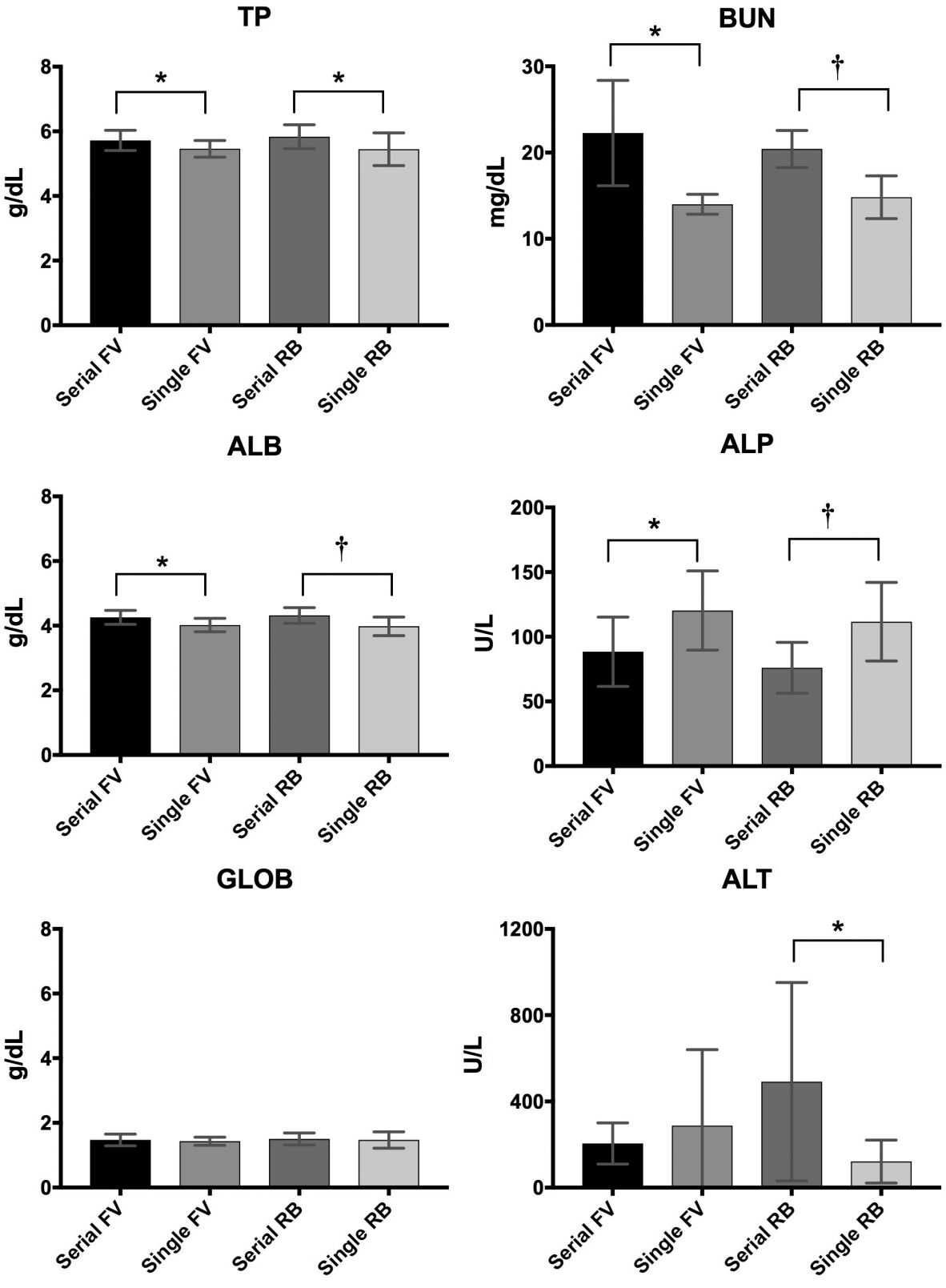

Figure 8. Serum chemistry values across serial and single-collection RB and FV groups. Error bars indicate 1 SD; *, $P < 0.05$; †, $P < 0.01$.

study, some differences in the CBC results correspond with findings from previous studies, whereas others contradict previous findings. For example, neutrophils and RBC were significantly decreased in the RB groups relative to the FV groups, similar to previous findings.[15] Conversely, monocytes were decreased in the FV group relative to the RB group, opposite to the effect seen in a previous study.[15] Although the use of anesthesia in the RB groups might have affected the differences in cell populations between the 2 blood collection methods, previous authors concluded that blood collection site—and not anesthesia—is the

predominant variable that affects cell populations in the CBC.[15] In addition, differences in age or sex of mice evaluated might contribute to differences between CBC parameters in our study and others. Another interesting result is that during week 6, the numbers of WBC, neutrophils, and lymphocytes from both FV and RB groups were significantly decreased compared with all other weeks of blood collection. Because there was no significant difference between groups during this week, this decrease is unlikely to be related to the blood collection method. More likely explanations for this decrease are a natural age-related change and an artefactual decrease due to machine error.

Furthermore, clinical chemistry values and blood quality parameters can vary depending on the blood collection site.[1,4,7,23] We investigated whether the frequency of blood collection (single compared with serial) and the method of blood collection would influence clinical chemistry values. To eliminate the blood collection site as a variable in the clinical chemistry analysis, we collected blood by using the intracardiac method at the end of the study for each group. Overall, no differences were detected between the single FV and single RB groups or between the serial FV and serial RB groups. However, statistically significant differences did appear when data were analyzed between a serial group and its respective single-collection group. This result suggests that these differences are due to either the increasing age of the animals or to the frequency of blood collection. For example, albumin and total protein were higher in serial groups, whereas globulins were approximately equal across all groups, indicating that the increases in total protein in both serial groups are related to increased albumin. Differentials for albumin increases include dehydration and aging.[18,28] Although the actual differences in the albumin values may not be clinically relevant (values stayed within normal ranges for all groups), the elevated albumin value seen in both serial FV and serial RB groups might indicate that these animals had a poorer hydration status, potentially due to weekly blood collection. In addition, age-related changes can account for the difference in albumin values, given that mice in the serial groups were 6 wk older than animals in the single-collection groups at the time of clinical chemistry analysis. Along these same lines, ALP levels were decreased in both serial groups relative to single groups, likely as a result of the age difference given that ALP levels drop due to osteoblastic activity associated with rapid growth after 3 mo of age.[18] Mean ALT levels were the only clinical chemistry values that were clearly outside of the normal reference ranges in all study groups, likely due to hemolysis of the samples,[17] given that there were no increases in other liver enzymes (ALP, AST) and that none of the mice displayed clinical signs of liver disease.

Considering the incidence of mortality, the severity of histologic lesions, and occurrence of clinical adverse events such as convulsions, we conclude that the FV method is less desirable for serial blood collection in mice compared with the RB method. The etiology of these mortalities is still unclear, although the presence of cerebral trauma in 2 of the mice that died suggests that the FV method has the potential for more severe trauma to critical organs as compared with the RB method. With regard to the collection of a single blood sample, measured clinical and welfare parameters did not differ between the FV and RB methods. As expected, serial blood collection, regardless of method, was associated with increased incidence of histopathologic lesions compared with single-collection groups, although the mild increase between single and serial RB groups suggests that serial RB collection does not impute a significantly higher risk of tissue damage. Future studies should consider whether other blood collection techniques, such as from the FV with anesthesia or from the lateral saphenous vein, might present even better options as serial blood collection techniques in mice.

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