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Type, Duration, and Incidence of Pathologic Findings after Retroorbital Bleeding of Mice by Experienced and Novice Personnel

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Retroorbital blood collection is a common technique in laboratory rodents due to the ease with which it can be performed and the sample volumes obtained for subsequent blood analyses. However, its use has been discouraged recently due to aesthetic discomfort and anecdotal reports of potential for ocular injury during blood collection. We hypothesized that a single standardized session of in-person training would be sufficient to learn the appropriate technique and minimize the likelihood for adverse outcomes. Experienced instructors (n = 2) conducted hands-on training classes to teach novice personnel (n = 40) to perform this procedure. Blood was collected from anesthetized mice (n = 40) via a capillary tube first placed at the medial canthus of the right eye and then advanced into the retroorbital space; the left retroorbital spaces served as unmanipulated controls. For comparison, the experienced instructors similarly collected blood from 40 additional mice. The tube could be inserted only once in each mouse, with the goal of obtaining 50 to 100 μL blood. Overall, 79 of 80 mice (98.8%) showed normal body condition, posture, and behavior throughout the 14-d study. Thus, any clinical observation scores pertained specifically to ocular lesions, which occurred at least once after sampling in 43 (53.8%) of the mice. Clinical and histopathologic scores of mice after bleeding did not differ between experienced and novice personnel. We conclude that a coordinated hands-on training program can provide consistent and sufficient instruction for research personnel to conduct retroorbital blood collection with competence in anesthetized laboratory mice.

Abbreviation: ULAR, University Laboratory Animal Resources.

Veterinary staff at our institution presumed that findings of spontaneous and nonspecific ocular lesions were potentially secondary to incorrect or inexperienced retroorbital blood collection. Given our robust training program for scientific staff in rodent handling and clinical techniques, we wanted to determine whether this presumption was accurate, because mice often present with ocular lesions (including periocular swelling, conjunctivitis, blepharospasm, and opacities of the globe) secondary to aspects of the cage microenvironment, husbandry parameters, trauma, or fighting with cage mates. In fact, nonspecific ocular lesions were found to be the 4th highest reported clinical issue in our mouse colonies.[32] The current study was designed to test assertions that this classic blood-sampling route leads to unacceptably high numbers of adverse ocular outcomes and to verify that trained staff members are capable of competently performing the sampling procedures.

Historically, retroorbital sampling has commonly been used to collect blood from laboratory mice and is endorsed in IACUC-approved guidelines at our institution. This method allows for rapid, consistent collection of moderate blood volumes from the venous plexus located in the retrobulbar space (the area behind the globe of the eye). Precise anatomic descriptions of the retroorbital sinus of the mouse are limited, but it has been depicted as an ample blood source due to the confluence of several vessels, likely including the supraorbital vein, inferior palpebral vein, dorsal nasal vein, and the superficial temporal veins.[56]

In recent years, the retroorbitaltechnique has come under increased scrutiny due to the perception that it is aesthetically displeasing and due to anecdotal animal welfare concerns. In some countries, these concerns about humane use have led to the abolition of retroorbital bleeding as a route of blood collection in favor of other sampling approaches, like the submandibular plexus and saphenous and tail vein sites.[19] In reality, each method of blood sampling in mice is associated with some degree of precaution and a potential for adverse outcomes, given the small size of the laboratory rodent, challenging blood-access sites, and total blood volume limitations.

Explicit and uniform guidelines have not been established for retroorbital sampling to maximize blood collection and minimize potential for adverse outcomes. In the United States, the retroorbital procedure is still widely used, although guidance on the performance of retroorbital bleeding varies widely by institution.[10,12,28,34,35] Although the collection procedure itself is brief, it involves the intentional breach of vasculature integrity with the potential for discomfort and subsequent retrobulbar hemorrhage that must be abated. Due to the placement of the collection device into the retrobulbar space, most institutions require that mice are anesthetized to remain immobile (and thus minimize additional trauma due to conscious movement) during the procedure.[5,10,28,34,52] Institutions that do not require general anesthesia for retroorbital sampling instead most often mandate the use of topical ocular anesthetics, such as proparacaine hydrochloride, to be applied to the eye prior to collection.[28,35]

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In addition, when subsequent blood draws are necessary, various authors recommend that collection from the retroorbital space should be alternated between right and left eyes and that consecutive (repeat) samplings from the same eye should be avoided until a recovery period has elapsed.[10,28,52] Recommendations for recovery periods between repeated blood draws from the same eye vary widely, ranging from no explicit guidance[12] to indications that 10 to 14 d[5,10,28] or as long as 28 d[52] should elapse between repeat retroorbital draws from the same eye. Although blood volume typically returns to prevenipuncture volume within 24 h of blood collection in mice, some parameters (including erythrocyte counts) require as long as 14 d to rebound to prebleed levels.[25] Recent data from a study in C57BL/6 mice indicated that a maximum of 15% of the blood volume of male mice and as much as 25% of blood volume of female mice can be collected once weekly for 6 wk with no adverse effects and with full recovery of hematologic parameters during the week between samplings.40 In addition, there is no consensus regarding the maximal number of blood samples that can be collected from the same eye, with recommendations varying from no repeat collections at all[17] to a defined number permitted[10,28] to no limitation of number of retroorbital bleeds performed.[35]

Given the lack of consensus regarding retroorbital sampling, we suggest that current institutional and global guidance may be based more on anthropomorphic interpretations rather than on scientific investigation of the effects of this sampling method. To our knowledge, no studies in laboratory mice have investigated the incidence, frequency, and duration of lesions after retroorbital bleeding or have compared phlebotomists with different experience levels. We hypothesized that participation in a structured mouse handling and procedures training class would prepare attendees to master the retroorbital blood sampling technique and ultimately prevent severe ocular lesions, similar to the competence shown by experienced phlebotomists. The results of this study will lead to the development of evidence-based welfare recommendations regarding the frequency of retroorbital blood collection, anticipated tissue responses, and timing of repeat retroorbital samplings.

Materials and Methods

Animals. Clinically healthy retired breeder mice (n = 87; 80 females, 7 males; age, 4 to 6 mo; originally procured from NCI:NIH but bred inhouse for 10 to 20 generations) were donated and maintained on an approved IACUC protocol in housing conditions compliant with AAALAC accreditation and meeting the room humidity and temperature expectations for the *Guide for the Care and Use of Laboratory Animals*.[27] Mice were on a C57BL/6N background with a mutation in MDA5, which has been shown to be important in response to viral infection and replication but with no other demonstrated effects on ocular health.[18] All animals were housed under a 12:12-h light:dark cycle at a maximal density of 4 mice per static polycarbonate microisolation cage (Max 75, Alternative Design, Siloam Springs, AR) on disposable bedding (diameter, 0.12 in.; Bed-O-Cobs, Animal Specialties and Provisions, Quakertown, PA). Wire-lid food hoppers in cages were filled to capacity weekly with rodent chow to enable free-choice feeding (Lab Diet 5010, Animal Specialties and Provisions), and water was supplied by bottle. The housing room was classified for Animal Biosafety Level 1 (ABSL1) experiments. The mice were housed in a barrier facility and were donated as surplus naïve mice to the University Laboratory Animal Resources (ULAR) training group. The mice were transferred from an IACUC-approved research protocol to the ULAR training protocol according to institutional guidelines. The design of this study required mice to be transported to the dedicated training classroom and back to the facility to await experimental endpoints. To be relocated between the facility and the classroom, as directed by institutional policy, all cages were confirmed to be free of pinworms (*Syphacia obvelata* and *Aspiculuris tetraptera*) by testing a pooled fecal sample from each cage as well as performing perianal tape tests on 3 mice per cage.

Female Swiss-Webster sentinel mice (younger than 6 mo) at our institution were tested inhouse at both the barrier facility of origin and the destination conventional housing facility quarterly for 3 quarters each year and were found to be free from fur mites and pinworms (*Syphacia* spp. and *Aspiculuris* spp., by cecal exam). Sentinel mice also tested negative for antibodies to pathogens including: mouse hepatitis virus, mouse parvoviruses, rotavirus, *Ectromelia* virus, pneumonia virus of mice, Theiler murine encephalomyelitis virus, and Sendai virus. For one quarterly test each year, sentinels from each housing facility were tested by an outside contract laboratory and were found to be free from all pathogens contained on a comprehensive assessment panel (HM Assessment Plus panel, Charles River Laboratories).

Retroorbital blood collection and clinical monitoring. The mice provided to the retroorbital study were incorporated into routine institutional training classes for investigators to learn the basics of mouse handling and relevant procedures. Prior to retroorbital blood collection, mice were randomly assigned into experimental groups (n = 2) to be bled by either a novice or an experienced phlebotomist (n = 40 mice per group) and to an experimental endpoint (day 0, 1, 3, 7, or 14 after bleeding; n = 8 mice per endpoint per group). Ear punches were used to individually identify mice. A target sampling range of 50 to 100 µL of blood was collected from each mouse, per the technique instructions provided by the ULAR Training course and as directed by the experienced instructors (described following). All retroorbital bleeding was performed on anesthetized animals (as described later), which were retained for subsequent ocular and periocular evaluation.

Experienced instructors (n = 2) were ULAR Training course leaders (one of whom is a licensed veterinary technician with LAT certification and the other of whom has LATG certification); each has more than 5 y of experience in performing the retroorbital blood collection technique. For the mice assigned to these experienced phlebotomists, 2 sessions were held during which each instructor bled 10 mice (n = 20 mice total per session, 40 mice total in the experienced group). By using an anesthetic vaporizer (RC-2 Rodent Circuit Controller, VetEquip, Pleasanton, CA), mice were anesthetized by isoflurane inhalation in an induction box (1-Liter Chamber, product no. 941443, VetEquip, Pleasanton, CA) at 2.5% to 3% and maintained at 1% to 2% isoflurane through a nosecone (Bubble Tubing Nosecone—small to medium mouse [9 mm], product no. 921609, VetEquip) on a Bain nonrebreathing circuit (Pedi-Bain 45-in., product no. 921410, VetEquip). The mice were confirmed to be at a surgical plane of anesthesia through the lack of a pedal reflex (no response to a firm toe pinch).

The 2 experienced trainers then collected blood via an approach at the medial canthus by using a 100 µL capillary tube (Microhematocrit Tube, Nonheparinized, Jorgensen Laboratories, Loveland, CO) from the retroorbital sinus of the right eye exclusively. Blood collection was performed as described previously.[25] Briefly, anesthetized mice were placed in left lateral recumbency on the tabletop. The head was grasped and the eyelids gently retracted to cause protrusion of the globe of the

right eye. The capillary tube was inserted at the medial canthus at a 45° angle directed in a caudomedial direction toward the middle of the eye socket. The tube was rotated, and gentle downward pressure was applied through the conjunctiva at the medial canthus until the retroorbital sinus was entered and blood flowed into the tube (Figure 1).

The capillary tube was inserted only once into the sinus of each mouse. The volume of blood collected within the capillary tube was recorded for all except 4 of the study participants, to control for variability of capillary tube placement and to determine competence at performance of this technique. In applicable cases, the estimated volume of blood that welled around the medial canthus but did not flow into the tube was recorded also. These estimated volumes were obtained by measuring the volume of a comparably sized drop of water, to avoid additional manipulation of the eye that would have been required to directly measure the volume of free blood. The nonsampled left eye served as a control and precluded the need for an additional intracage control animal. After blood was drawn, the palpebrae of the right eye were closed gently and a 4 × 4-in. gauze was applied with light pressure for 30 s to ensure hemostasis. The mouse then was returned to a clean cage placed on a warm recirculating-water blanket and monitored until fully recovered from anesthesia. Mice assigned to the day 0 endpoint were allowed to recover completely from anesthesia and then were taken for euthanasia and tissue collection within 120 min of the end of the procedure. All other mice that were assigned endpoints after day 0 postbleed were returned to the housing facility for clinical monitoring and assessment until their assigned experimental endpoints.

Novice handlers (n = 40) were employees of the institution that intended to participate in animal research protocols after receiving in-person training in rodent handling and procedures. Predetermined criteria for inclusion as novice personnel were that the person had not attended the training class previously and had not conducted retroorbital sampling on mice for at least 12 mo prior to the training session in which they were enrolled. In actuality, the novice handlers had never performed retroorbital sampling previously, and most of them had never handled mice prior to the training class. The novice personnel attended the training class and performed all procedures presented in the training class with the exception of the retroorbital bleed. These novice personnel then observed a retroorbital bleed performed by one of the experienced trainers, with step-by-step oral instructions given immediately preceding the procedure. The novices then performed the retroorbital procedure according to instruction from the trainers.

All mice were monitored for clinical and ocular effects after sampling. Every mouse with an endpoint past day 0 was evaluated on day 1 after sampling and then intermittently until a final clinical assessment immediately prior to euthanasia at the assigned endpoint. Each mouse was evaluated for activity level and demonstration of appropriate species-specific behaviors, posture, and body condition score (scale of 1 [extremely thin] to 5 [obese]).[51] As well, both the left and right eyes were scrutinized grossly to detect the presence of ocular lesions including discharge, redness, visible scabbing or continued evidence of bleeding, swelling, opacity of the cornea, or blepharospasm. Ocular lesions were scored on a predetermined scale of 0 (no lesions) to 3 (severe lesions including discharge, marked edema, and blepharospasm). Humane endpoint criteria were established such that any mouse demonstrating overt signs of pain or distress (severe ocular signs or self-trauma with hunched posture and diminished activity) would have been removed from the study.

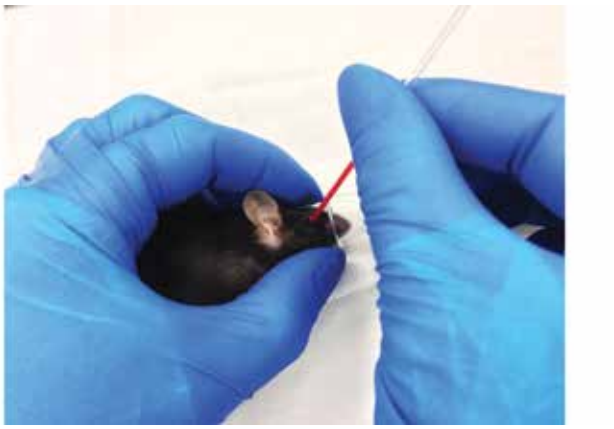

Figure 1. Method for collection of blood from the retroorbital sinus. Mouse under isoflurane anesthesia is in lateral recumbency with the nosecone supported to maintain anesthesia during the procedure.

The study design included the assignment of a total of 80 mice for retroorbital bleeding; however, during enrollment, 6 mice were disqualified because of spontaneous ocular lesions noted prior to initiation of the study. An additional animal was disqualified because a novice phlebotomist mistakenly bled a training mouse before performing the retroorbital bleed on the study mouse, thus no longer meeting our definition of a novice. These disqualified mice (n = 7) were replaced with comparable surplus mice of the same age and strain.

Tissue preparation and histopathologic scoring. Euthanasia was performed via CO_2 narcosis, and death was confirmed by the creation of a bilateral pneumothorax by thoracotomy with sharpened scissors. After confirmation of death, photographs were taken of each eye of each mouse to document ocular health status. The head was handled carefully to avoid accidental postmortem trauma artifacts to the eye or associated structures. Heads were submerged in 10% neutral buffered formalin. After at least 48 h of fixation, heads were submitted to the histopathology lab at the Veterinary Hospital of the University of Pennsylvania for decalcification overnight in 15% formic acid. Half of the 80 total heads in the study were selected for histopathologic analysis, with 4 being selected from each time point (0, 1, 3, 7, and 14 d) of both the novice and experienced sets of mice. This assignment resulted in 10 groups with 4 heads analyzed from each group. Heads (n = 40) were selected, after consultation with a board-certified veterinary pathologist and with a statistician, to represent the full range of clinical scores and blood volumes collected. Random selection of samples was not pursued due to the potential skewing of the data if selection did not include the full complement of potential clinical scores from each group. This decision was made based on the consideration that clinical scores potentially reflected underlying histopathology. Each decalcified head was trimmed into right and left sides, and each half was embedded separately in paraffin, with the medial side down. Sagittal sections (10 µm) were taken through each eye to allow separate blinded scoring of the bled (right) and control (left) eye from each mouse; specifically, sections for assessment were taken from the medial canthus as well as the central portion (including optic nerve) of each eye. Slides underwent routine staining with hematoxylin and eosin. In consultation with a board-certified veterinary pathologist blinded to the groups, the central sections were analyzed and scored for presence and severity of microscopic changes. Each

eye was scored on a scale of 0 to 3 (0, none to minimal; 1, mild; 2, moderate; 3, severe) for 2 different pathologic changes, microscopic hemorrhage and inflammatory infiltrate. Inflammation was scored independent of the specific inflammatory cell type present. Scores were compared between the bled and control eye for each mouse as well as compared across experimental groups, phlebotomists, and time points.

Statistical methods. We summarized the clinical scores, microscopic hemorrhage scores, and inflammation scores assigned to each animal according to time after sampling. To evaluate differences between the novice and experienced groups at each time point, we performed a Fisher exact test for comparing the frequency distribution of scores, a 2-group *t* test for comparison of means, and a Wilcoxon rank test for comparison of medians. To test the clinical, microscopic hemorrhage, and inflammation score changes over time and the difference between novice and experienced groups, 2-way ANOVA with repeated measures were performed, followed by posthoc pairwise comparisons.

We also summarized histopathologic scores for both microscopic hemorrhage and inflammation in the tissues surrounding the right compared with left eye and for novice compared with experienced groups. To evaluate differences between the novice and experienced groups at each time point, we performed the Fisher exact test to compare the frequency distribution of scores, 2-group *t* test for comparison of means, and Wilcoxon rank test for comparison of their medians. To evaluate differences in microscopic hemorrhage and inflammation between the left and right eyes, we performed paired *t* tests.

All statistical analyses were performed in SAS (version 9.s, SAS Institute, Cary, NC), and *P* values less than 0.05 were considered to be statistically significant.

Results

Volume of blood collected. Because only one insertion of the capillary tube was permitted according to our study parameters, we recorded both the volume of blood collected within each participant's capillary tube as well as an estimate of the volume of any blood that was present at the medial canthus but did not flow into the tube. Novices collected an average of 53.9 µL into the tube, with an additional average 18.3 µL that did not flow into the tube. Experts collected an average of 79.7 µL into the tube, with an additional average 4.3 µL that did not flow into the tube.

Clinical scores. Mice were assigned a clinical observation score ranging from 0 (normal clinically) to 3 (severe general or ocular lesions) at intervals between blood collection and randomized endpoint, on the basis of overall body condition, posture, activity level, and demonstration of appropriate species-typical behavior and the specific presence of ocular and periocular lesions, including discharge, swelling, corneal opacity, and blepharospasm (Figure 2). Overall, 79 of 80 mice (98.8%) enrolled in the study displayed clinically normal body condition, posture, and behavior at every clinical check throughout the duration of the study, such that, for all intents and purposes, the clinical observation score pertains specifically to ocular signs. The single mouse to exhibit systemic clinical signs displayed normal body condition with slightly hunched posture and a decreased activity level on day 1 after sampling but received a score of 0 before its assigned endpoint on day 3. The score assigned to this mouse according to both ocular and systemic clinical signs was the same as that assigned based on ocular signs alone. No mouse demonstrated humane endpoint criteria, as described previously, and all reached their assigned endpoints.

Clinical scores were highest at the earliest time points after sampling and decreased over time, as we anticipated, with the greatest incidence of visible ocular lesions occurring until day 3 (Figure 3). Of the ocular lesions we selected to record, blepharospasm was the most common clinical sign observed and occurred in the right eye of 32 of 80 mice (40%). The second most common clinical sign was serous discharge, which affected the right eye of 24 of 80 mice (30%). Less common clinical signs seen in the right eye included mucoid discharge (9 of 80, 11.3%), periocular alopecia (6 of 80, 7.5%), periorbital edema (5 of 80, 6.3%), nictitating membrane inflammation (3 of 80, 3.8%), mild exophthalmia (2 of 80, 2.5%), mild corneal opacity (1 of 80, 1.3%), and scab on eyelid (1 of 80, 1.3%). Clinical signs observed in the left control eye included blepharospasm (2 of 80, 2.5%), mucoid discharge (1 of 80, 1.3%), and nictitating membrane inflammation (1 of 80, 1.3%). In total, 37 of 80 mice (46.3%) had no grossly observable clinical abnormalities during the course of the study, 2 (2.5%) had clinical abnormalities of the left eye only, 2 (2.5%) had clinical abnormalities of both eyes, and 39 (48.8%) had clinical abnormalities of the right eye only. Of the 43 mice with clinical ocular abnormalities, 18 (41.9%) had a single, isolated ocular lesion whereas the other 25 (58.1%) had some combination of clinical signs.

Of the 16 mice with a day 14 endpoint, 13 (81.3%) had no clinical signs at day 14, whereas the remaining 3 (18.8%) had visible ocular abnormalities. Of the 3 mice with ocular lesions at day 14, 2 were ongoing issues throughout the study, whereas the 3rd had no clinical abnormalities until mild ocular discharge was seen on day 14. Of the 13 mice with no clinical abnormalities at day 14, 7 were clinically normal throughout the entire 14-d observation period, whereas the other 6 initially had clinical ocular abnormalities that resolved before day 14.

The overall improvement in clinical outcomes over the duration of the study was exemplified by significant decreases in the clinical score between days 0 and 7 ($P < 0.001$) and between days 0 and 14 ($P = 0.02$). When clinical scores at endpoint were compared, the morbidity of mice bled by novices did not differ compared with those bled by experienced phlebotomists (Figure 4). Multiple statistical analyses found no difference in clinical scores of mice between the novice and experienced groups at any of the time points examined ($P > 0.30$ for all comparisons) or with all time points combined ($P = 0.78$).

Microscopic hemorrhage and inflammation. For the subset of 40 mice selected for histopathologic analysis, microscopic hemorrhage and inflammation scores were assigned to both the sampled right eye and the unmanipulated (control) left eye by a pathologist naïve to the assigned groups and to the clinical histories. Microscopic evidence of hemorrhage was found in numerous structures around and within the orbit, including the retroorbital sinus, nasolacrimal duct, periocular musculature, and Harderian gland. Cellular characteristics contributing to the inflammation score included documented inflammatory infiltrate, the components of which varied depending on the time point. Specifically, predominantly neutrophilic infiltrates were present at days 0 and 1, predominantly neutrophilic infiltrates with some areas of mixed cells (neutrophilic and lymphoplasmacytic with fewer macrophages) were apparent on day 3, predominantly mixed (neutrophilic and lymphoplasmacytic with fewer macrophages) with some areas of mononuclear infiltrates (lymphoplasmacytic with fewer macrophages) were revealed on day 7, and predominantly mononuclear infiltrates (lymphoplasmacytic with fewer macrophages) found at day 14. The extent and severity of inflammation was evaluated for

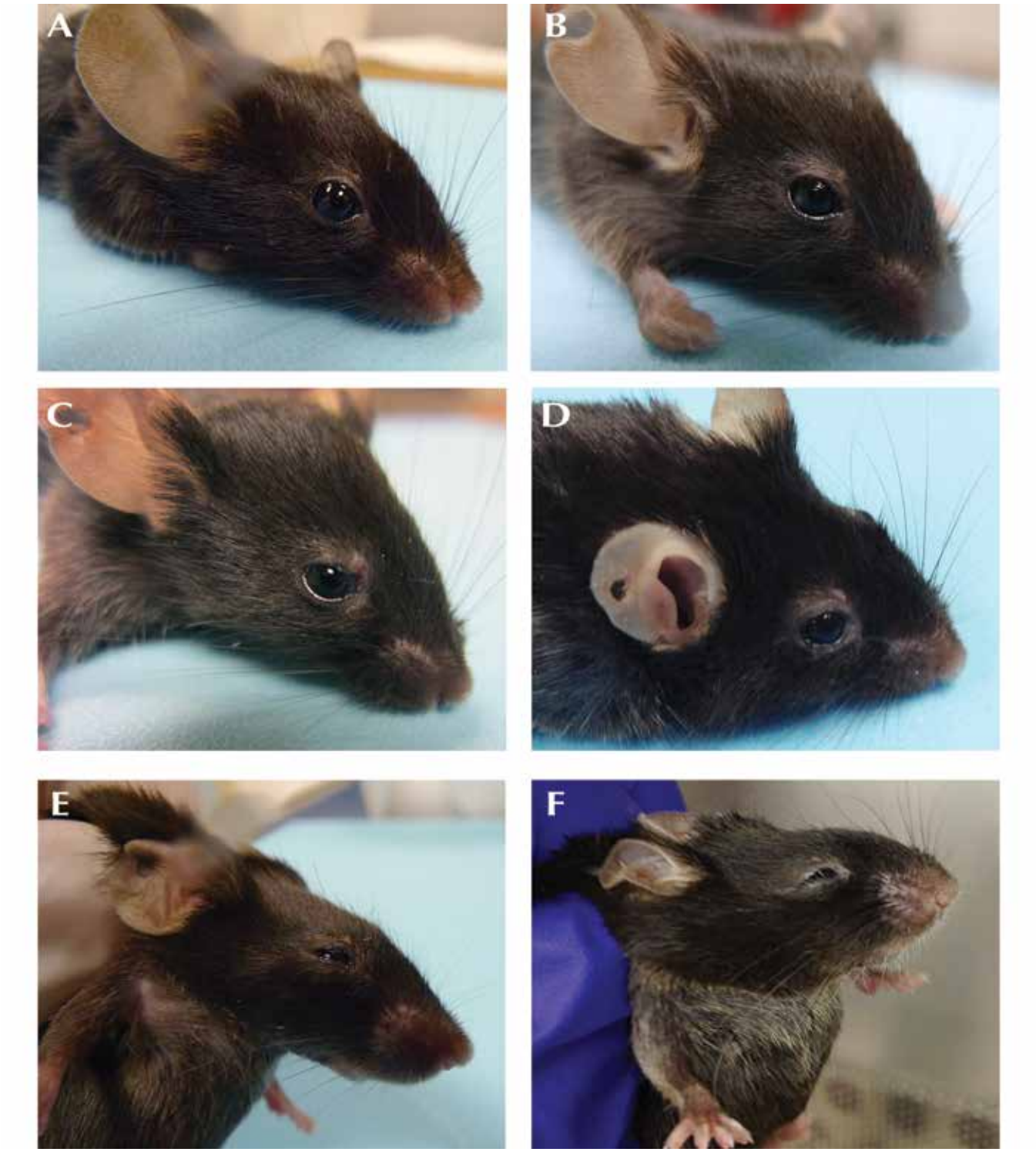

Figure 2. Representative images of mice receiving a clinical score of (A) 0, (B) 1, (C and E) 2, or (D and F) 3. Scores correspond to severity of ocular signs, ranging from 0 (no abnormal ocular signs) to 3 (marked edema and blepharospasm with or without mucoid discharge). Also included in the clinical score were body condition, posture, behavior and activity, and left eye clinical signs.

the inflammation score. Inflammation was found in the same range of ocular and periocular tissues as was noted for hemorrhage. Inflammation infiltrating the periocular skeletal muscle or Harderian gland (or both) was occasionally present, with degeneration and necrosis of the tissue contributing to the lesion severity and score. Occasional broken hair shafts were found in the periorbital tissue, often surrounded by neutrophils. Notably absent for all sections analyzed were any microscopic signs of hemorrhage or inflammation within the orbital bone or within or surrounding the optic nerve (Figure 5).

We found no significant differences between severity of microscopic lesions caused by retroorbital blood collection performed by experienced compared with novice phlebotomists (all *P* values greater than 0.60; Figure 6). In addition, microscopic hemorrhage or inflammation scores did not differ between novice and experienced phlebotomists at any time point (all

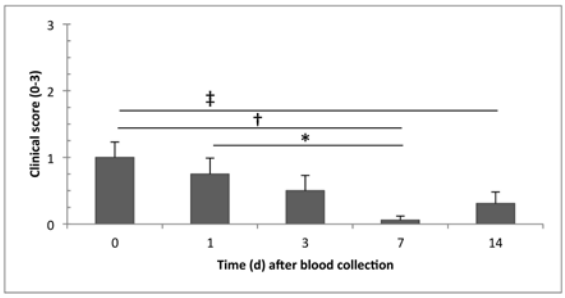

Figure 3. Clinical scores significantly improved over time in all groups, with pairwise analysis showing statistically significant improvements between days 0 compared with 7 (†, $P < 0.001$), 0 compared with 14 (‡, $P = 0.02$), and 1 compared with 7 (*, $P = 0.006$).

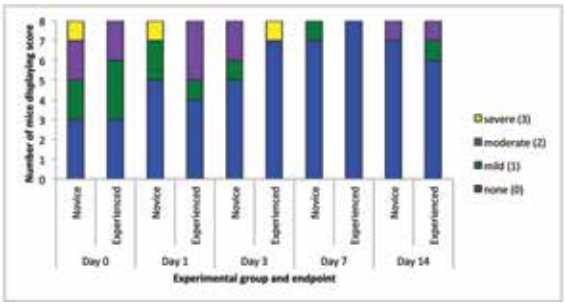

Figure 4. Comparison of clinical scores at endpoint demonstrating no significant differences between novice and experienced groups at any time point. Data were compared by using the Fisher exact test, 2-group *t* test for comparison of means, and Wilcoxon rank test for comparison of medians. All *P* values exceeded 0.30 (cutoff for significance, 0.05).

P values greater than 0.32, data not shown), A full range of scores was found for both microscopic hemorrhage and inflammation in the sections from the sampled (right) eyes, with a similar distribution of scores between groups (Table 1).

Because of the lack of significant differences between the novice and experienced groups, the groups were pooled for statistical analysis of changes in microscopic hemorrhage and inflammation scores over time. As was seen for clinical scores, scores describing microscopic hemorrhage improved over time (Figure 7), with significant differences between days 0 and 3 ($P = 0.005$), days 0 and 7 ($P < 0.0001$), days 0 and 14 ($P = 0.007$), and days 1 and 7 ($P = 0.002$). All other pairwise comparisons did not demonstrate a significant difference. Inflammation scores were higher on day 1 compared with day 0 ($P = 0.005$) but then decreased over time (Figure 7), with significant differences between days 1 and 7 ($P = 0.0002$), days 1 and 14 ($P < 0.0001$), and days 3 and 14 ($P = 0.02$). All other pairwise comparisons were nonsignificant.

For all groups, as expected, the bled eye had a significantly higher inflammation score than did the control eye (Figure 8; $P < 0.0001$). In addition, microscopic hemorrhage scores were higher in the right eye compared with the left in the experienced group ($P = 0.008$) and trended higher in the novice group ($P = 0.07$).There was no concordance in microscopic hemorrhage or inflammation scores between the left and right eye, indicating that the unexpected finding of microscopic hemorrhage around the left eye was not correlated to a high hemorrhage score in the right eye of the same mouse.

Discussion

During professional veterinary discussions of clinical cases at our institution, an assumption was made that spontaneous and nonspecific ocular lesions noted in our mouse colonies were likely linked to complications associated with retroorbital sampling by untrained personnel. We compared novice and experienced phlebotomists to determine whether there was a difference in outcomes for mice after retroorbital blood sampling. In fact, our results indicated that training through an in-person hands-on class, such as the one required of all personnel listed on animal protocols at our institution, is sufficient to teach novices retroorbital blood collection in a way that produces competency of technique when compared with outcomes obtained by experienced staff. We defined competency in this technique to mean that, after a demonstration, the participant was able to collect at least 50 µL of blood from the retroorbital sinus of a mouse without increased adverse outcomes for the study animal. All novices involved in this study successfully accessed the retroorbital sinus and collected blood. With the single insertion of the capillary tube permitted in this study, 50% of novice personnel were able to collect at least 50 µL of blood within the tube; if redirection of the tube had been permitted by our study parameters, our estimates of additional blood outside of the tube indicate that 86% of novices would have collected at least 50 µL blood. Experienced personnel obtained at least 50 µL of blood in the tube 88% of the time. If the experienced personnel had been permitted to redirect the tube, our estimates of additional blood volume indicate that at least 50 µL would have been collected 93% of the time. The greater success of experienced compared with novice personnel in obtaining flow of blood into the tube with a single insertion is the criterion that we used to distinguish proficiency from competency in this technique. The fine-tuning of the dexterity required to manipulate the capillary tube to optimize flow of blood into, rather than around, the tube is an example of a skill that will develop with additional use of this technique outside of the training class.

In the hands of a trained operator, adverse complications from retroorbital bleeds are rare and distress to the animal is minimal due to care taken during anesthesia and handling. Training is an essential component of any animal care and use program and often involves a combination of various training methodologies, including online and in-person sessions followed by either self-assessment or completion of a training checklist and evaluation by course instructors. The institution should provide this training to researchers, including certain key concepts for all, as well as tailoring the topics for specific researchers' needs to ensure sufficient training before beginning animal work.[27] Competency is established by evaluating the trainee's ability to perform the task during the training session, whereas proficiency is established through follow-up evaluation after the trainee has had time to practice and refine the technique.[8] Although the hands-on training class at our institution achieves competency, the current study did not verify proficiency after class completion. This confirmation would be an interesting future study topic, especially when considerable time elapses between the completion of the course and actual independent performance of the task in a research setting. Our institutional online training course is, however, accessible to researchers at any time as a refresher module and includes written instructions on retroorbital blood collection.

Many of the recommendations for training methodologies in veterinary technical skills stem from the human surgical training literature. There is general agreement that technical skills are better learned in hands-on laboratory or simulation environments rather than remotely, but the number of repetitions required to achieve proficiency in clinical skills varies.[14] Considering the lack of differences in ocular complications seen

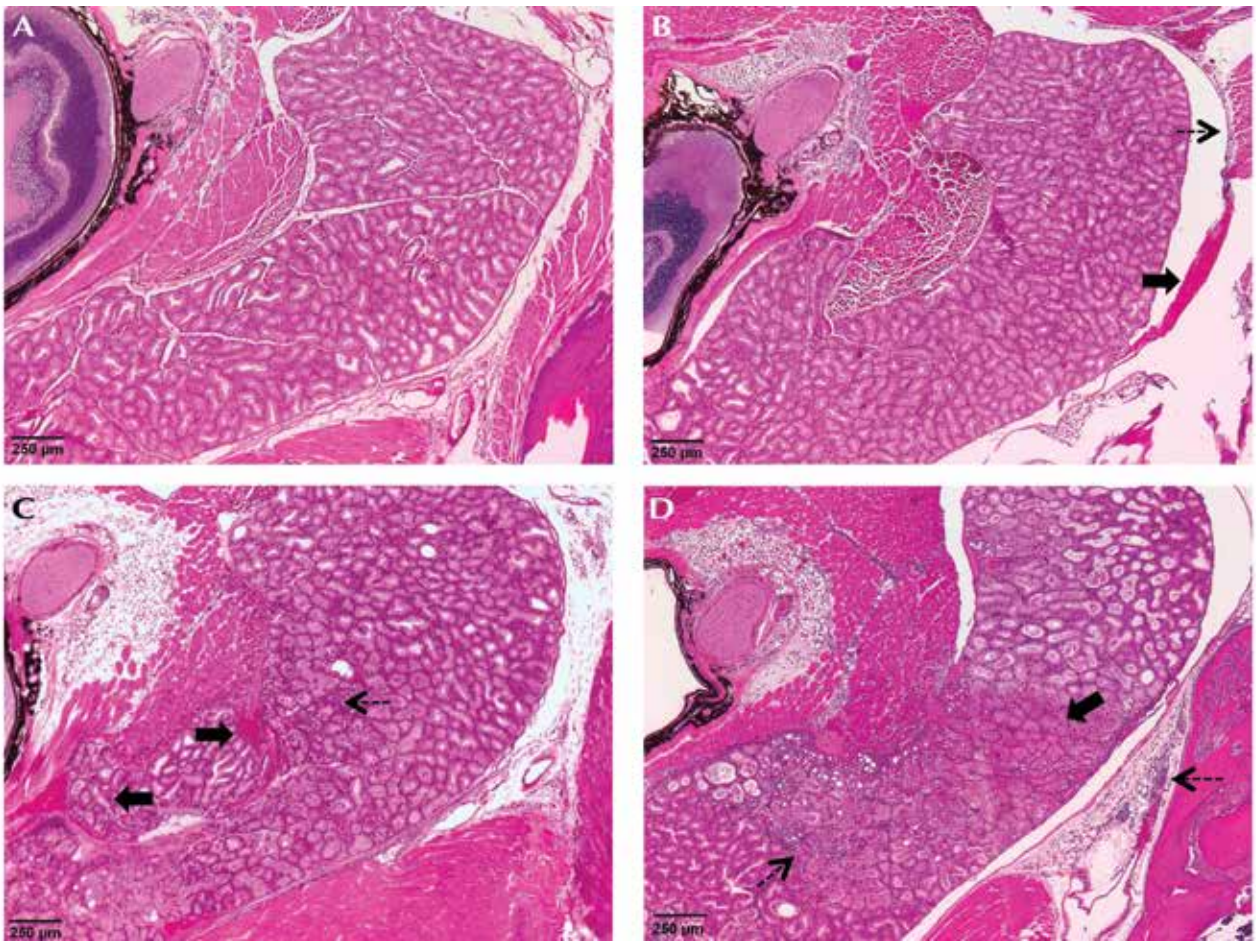

Figure 5. Sagittal sections demonstrating scores of (A) 0, (B) 1, (C) 2, and (D) 3 for both microscopic hemorrhage (solid arrows) and inflammation (dashed arrows) in each micrograph. Hematoxylin and eosin stain.

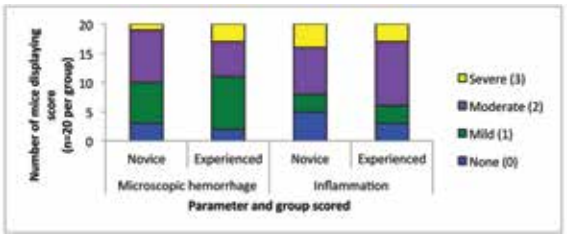

Figure 6. Comparison of histopathologic scores of the right (bled) eye at endpoint demonstrating no significant differences between novice and experienced groups. Data were compared using Fisher's exact test, 2 group *t* test for comparison of means, and Wilcoxon rank test for comparison of medians. All *P* values exceeded 0.60 (cutoff for significance, 0.05).

in mice bled by experienced compared with novice personnel in this study, we suspect that whereas complicated surgical procedures may require many repetitions to achieve peak performance, proficiency in a relatively uncomplicated technique, such as retroorbital bleeding, may be achieved expeditiously with instruction and subsequent practice.

Our definition of novice personnel led to some study limitations, because only handlers who had never performed the retroorbital technique were eligible. We had no difficulty in finding eligible participants among the training-class students, because none of our novice study participants had performed retroorbital bleeding previously, and many had never handled a mouse prior to the class. Because of the size of the animal use program at our institution, with an average of 600 students enrolling in the hands-on mouse training program annually, there was a wide pool of study candidates from which to draw. The limitation arose first in that only the first attempt by any student in the class would be eligible for analysis in this study, resulting in the disqualification of a single mouse when a student mistakenly performed retroorbital blood collection on a training class mouse prior to working with the assigned study mouse. In addition, the amount of blood collected by novice phlebotomists was difficult to record, because our study parameters only permitted one insertion of the capillary tube. The more tentative approaches taken by the novice personnel led to several students who successfully punctured the retroorbital sinus but then had difficulty directing flow of the sample into, rather than around, the opening of the capillary tube, making accurate assessment of all collected volumes practically impossible. However, even considering this limitation, novices still collected an average of 53.9 µL into the capillary tube, a volume that is sufficient for many measurements. Under normal blood collection conditions, which permit the tube to be redirected and the collection of blood that welled around the tube by using a second tube or syringe, volumes approaching or exceeding the goal of 100µL could be obtained.

In this study, blood was collected from the right eye of all mice whereas the left eye served as a control. Although we found that the inflammation at the back of the right eye was consistently higher than that of the left, the data for microscopic hemorrhage were less clear. The mean periocular microscopic hemorrhage score for the right was significantly higher than the left for the experienced group (which we attributed to the greater volume

Table 1. Distribution of histopathologic scores in novice and experienced groups for left (control) and right (bled) eyes over all time points pooled

		Novice		Experienced	
	Histopathologic score	Left (n = 19)[a]	Right (n = 20)	Left (n = 20)	Right (n = 20)
Microscopic hemorrhage					
	0	31.6%	15%	30%	10%
	1	47.4%	35%	45%	45%
	2	15.8%	45%	25%	30%
	3	5.3%	5%	0%	15%
Inflammation					
	0	89.5%	25%	95%	15%
	1	10.5%	10%	5%	15%
	2	0%	45%	0%	55%
	3	0%	20%	0%	15%

[a]At the time of analysis, one sample was not returned after submission to the histopathology laboratory and was deemed lost from the assessment.

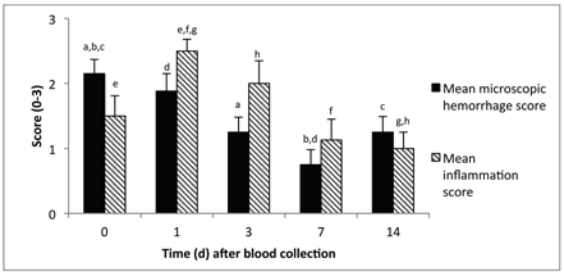

Figure 7. Microscopic hemorrhage scores improved significantly over time. Microscopic inflammation scores significantly increased on day 1 compared with day 0, and then significantly decreased over time. Error bars indicate standard error (n = 8 per bar, composed of pooled samples from both novice and experienced groups). Letters over bars indicate a significant difference between 2 bars with the same letter.

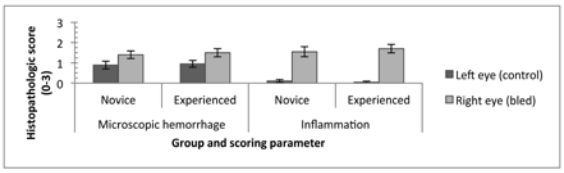

Figure 8. Comparison of mean histopathologic scores between right (sampled) and left (control) eyes. Eyes that were sampled had significantly higher inflammation scores than did control eyes. Microscopic hemorrhage score was significantly higher in the sampled eyes of the experienced group (P = 0.008) and trended higher in the novice group (P = 0.07). Data were compared by using the Fisher exact test. Error bars indicate standard error (n = 20 per group).

of blood collected by these experienced phlebotomists) and trended higher for the novice group, but unexpectedly, the left eye, across all groups, had microscopic hemorrhage scores as well. In other laboratory animal species such as rabbits, this phenomenon could be explained by the presence of an interophthalmic communicating artery;[16] however because this artery is not present in mice, the cause of the mild hemorrhage around the contralateral eye is unclear. There was no direct concordance of hemorrhage scores between the left and right orbits in each mouse, thus diminishing the likelihood that hemorrhage in the left orbit extends from that caused by blood collection from the right sinus, even if an undescribed communal vascular area exists between the retrobulbar spaces. One possible explanation is that left-sided hemorrhage was caused by unintended trauma from the table top, given that blood was collected from the right eye while the mice lay in left lateral recumbency. Further investigation is warranted into this phenomenon and may lead to changes in recommendations for positioning of mice during this procedure.

There are fewer published reports of lesions secondary to retroorbital bleeding for mice than for rats. One study in mice described Harderian gland necrosis and periocular muscular necrosis and hemorrhage at 6 d after retroorbital bleeding.[22] Lesions commonly listed as causing concern after blood collection from the orbital sinus in rodents include blindness, ocular ulcerations, puncture wounds, loss of vitreous humor, infection, and keratitis.[25,34] Interestingly, none of the studies cited earlier actually verified the presence of clinical blindness, despite the assertion that it was a side-effect of retroorbital bleeding. Concerns for blindness may originate from a single abstract that reported Wallerian degeneration of the optic nerve and retinal atrophy with 40% loss of retinal ganglion cells in rats after 15 mo of large-volume retroorbital blood collection every 6 wk.[30] However, clinical loss of visual acuity was not documented, and the relationship between retinal ganglion cell loss and reduction in visual acuity is complex and highly variable leaving some doubt as to whether this finding had clinical implications.[21] Studies in rats have noted increases in enophthalmia, ocular discharge, and corneal lesions after blood collection from the orbital plexus, with lesion incidence varying significantly between handlers (n = 4).[53,54] In contrast, a retrospective review of retroorbital bleeding procedures in rats showed that only 0.6% of 1869 rats bled via a lateral retroorbital access technique developed ocular injuries, and the samples obtained through the use of this method were of better quality than those obtained by saphenous venipuncture.[45]

The severity of these potential ocular problems contributed to our desire to verify whether the hands-on training program at our institution was sufficient preparation to instruct novice personnel to perform retroorbital blood sampling. In contrast to the suggestions of marked clinical deficiencies such as blindness and periorbital bone fracture after retroorbital blood collection, we did not observe any clinical abnormalities of this caliber, such as globe rupture, ocular infection, and fractures. The majority of our findings were histopathologic changes, including microscopic hemorrhage and inflammation within the periocular tissues. One of the most common sites of inflammation was the Harderian gland, often with epithelial cell degeneration and necrosis or less commonly periocular muscular necrosis. These findings are consistent with previous reports in mice.[22] Notably absent were any histopathologic indications of infection or of lesions that extended into the optic nerve or the bone of the orbit. The types of inflammatory cells we observed over time were consistent with the expected inflammatory response

after the exogenous induction of inflammation,[33] in this case, the tissue injury induced by retroorbital blood collection. A similar inflammatory response would be expected as part of the healing process in any location in which local tissue injury had occurred, such as at any site of blood collection. There were no differences in clinical or histopathologic outcomes between experienced and novice personnel, supporting the use of a hands-on training course as sufficient to limit unexpected outcomes after retroorbital blood collection. Both clinical and microscopic lesions appeared to peak within the first 3 d after blood collection and were less common and less severe by 14 d postcollection. This pattern lends support to maintaining at least 7 d between repeat retroorbital blood collections. Furthermore, adding alternation between eyes during subsequent collections would result in the bleeding of each eye once every 14 d. Thus sufficient time would be provided for the resolution of lesions before using the same eye for blood collection again.

The need for a prolonged recovery time between repeat samplings is a limitation of retroorbital blood collection and reduces the potential to use this method for studies requiring serial sampling. For studies that require more frequent sampling, other methods should be considered, including jugular cannulation[3] or catheterization,[38] jugular venipuncture on conscious mice,[46] and venipuncture of the tail, saphenous,[24] or dorsal pedal veins.[25] Of these, jugular cannulation or venipuncture yields the largest sample volumes. A recent report found jugular venipuncture in conscious mice to be a humane and practical method that allowed repeated collection of fairly large volumes with minimal adverse effects.[46] Smaller volumes (30 to 50 µL) can be obtained consistently from tail vein laceration or puncture or tail tip amputation, but even this smaller volume can be difficult to consistently obtain from the dorsal pedal vein.[7] Other studies found that repeated sampling of large volumes (maximum, 150 µL) can be obtained humanely through lateral tail vein incision in unanesthetized mice.[13,42] A modified dried-blood spot technique has been developed to offer additional refinement of blood collection techniques for research purposes and allows the use of a small sample from the lateral tail vein in pharmacokinetic studies rather than the large samples that were required previously.[29] From a welfare perspective, saphenous venipuncture and a tail-clip method have been found to be comparable.[1]

Several methods have been suggested as potentially humane alternatives to retroorbital blood collection, but results have been controversial. Submandibular venipuncture has been suggested as a preferable alternative to accessing the retroorbital sinus,[19,50] but other studies have found the method to cause significant morbidity under certain circumstances.[26] Typically, the facial vein is recommended only for one-time, nonserial collections.[9] Nude mice have been documented to be particularly susceptible to hematoma formation and tissue damage after sampling from the submandibular location.[37] Regardless of the technique selected, secondary hemorrhage (bruising), due to extravasation prior to achieving hemostasis, is likely after collection.

For pharmacokinetic or other studies requiring the serial collection of small volumes, a device such as a blood microsampling backpack[31] could be considered as a refinement to minimize the handling requirement for repeat sampling. The infrequently performed technique of sublingual blood collection in mice has been found to cause fewer pathologic changes than retroorbital blood collection[22] and has led to less tissue damage and improved food consumption and body weight gain than submandibular bleeding.[23] The drive to refine blood collection methods has been extended to other rodent species, such as the cotton rat (*Sigmodon hispidus*), in which the sublingual vein and subzygomatic facial venous sinus were both found to be humane alternatives to the retroorbital plexus.[2]

In addition to considering feasibility of obtaining the desired blood volume from a given venipuncture site, attention must be given to the numerous clinical pathologic and hematologic parameters, many of which show significant variation depending on the site of blood collection. Samples collected from the retroorbital sinus are more consistent over time than are tail vein samples,[55] but blood glucose and insulin values can differ between these sites.[7,41] Diverse other parameters vary depending on the blood collection site, including lipid profiles, markers of liver damage, catecholamine levels, and WBC counts.[6,15,20,36,43] These findings together demonstrate that blood collection site can have a significant effect on the parameters being studied and should therefore be taken into account when attempting to compare the results of disparate studies.

The specific methods used to perform retroorbital blood collection vary widely between practitioners and across institutions. In this study, mice were anesthetized by using isoflurane, which leads to rapid unconsciousness and consistently places mice at a safe and reversible surgical plane. Although a study performed on wild rodents indicated that survival after retroorbital blood collection was unaffected by the presence or absence of anesthesia,[11] we find the invasiveness of this procedure justifies the requirement for anesthesia for humane reasons in our rodent colonies. Previous studies claimed that topical proparacaine drops, when used as an adjunct to ketamine and medetomidine anesthesia, improved analgesia for retroorbital blood collection in mice.[49] However, the majority of mice in that study never lost their pedal reflex, indicating they may not have been at a deep surgical plane of anesthesia; in contrast, all mice in our study had a negative pedal reflex prior to blood collection and did not respond to the stimulus of retroorbital blood collection despite the absence of proparacaine. Isoflurane does not have residual analgesic effects, and given the higher clinical morbidity scores seen in study mice at early time points after collection, future experiments might warrant the addition of topical ocular analgesics as a complementary therapy to general anesthesia for the retroorbital collection procedure.

Although numerous studies have investigated alternatives for blood collection from the retroorbital sinus, this location still provides one of the best options for intravenous administration of larger volumes of experimental injections, including the installation of experimental compounds and reagents associated with bone marrow transplantation, leukemia induction, and gene therapy.[56] The other site commonly used for large-volume injections is the lateral tail vein, which several studies have found to be interchangeable with the retroorbital sinus in terms of efficacy.[39,44,48] Intravenous injection into the retroorbital sinus may be more humane than that of the lateral tail vein, given that repeated injection in the lateral tail vein results in behavioral changes consistent with a stress response whereas injection in the retroorbital sinus does not.[48] An additional benefit of injection into the retroorbital sinus is the increased consistency and accuracy of this procedure, which can result in reduced animal numbers.[44] Therefore, even when an institution chooses to discourage retroorbital blood collection in favor of the other sites discussed previously, accessing the retroorbital sinus will remain an important skill to teach in institutional training courses where personnel may require it for experimental intravenous injections.

The response to adverse conditions and disease processes shows many strain-specific variations, as do various blood parameters, including hemostatic factors.[4] The current study specifically looked at mice on a C57BL/6N background; therefore care should be taken in drawing exact correlations to other stocks or strains of mice. C57BL/6 mice are the most prevalent inbred mouse strain in use in research worldwide, lending wide applicability to the results of this study. This high prevalence of use also meant that many mice on this background were donated to our training protocol, allowing us to easily acquire a consistent study population. One unfortunate consideration in the use of C57BL/6 mice is their predisposition to numerous spontaneous ocular abnormalities, with reported incidence ranging from 4.3% to 10%.[47] An epidemiologic survey found that spontaneous ocular lesions account for 8.9% of clinical cases reported in mice of all strains at our institution.[32] In line with these previous reports, in the present study, 6 of 80 mice, or 7.5% of the animals enrolled initially, were reported to have spontaneous ocular lesions that were unrelated to retroorbital sampling but that led to their disqualification and replacement prior to initiation of the study. This high prevalence of spontaneous ocular lesions in laboratory mice, as well as the transient nature of gross clinical ocular abnormalities we observed after retroorbital blood collection, reinforces the inaccuracy of the presumptive attribution of eye lesions to retroorbital bleeding. These factors support that the vast majority of clinical cases of ocular abnormalities in mice are likely due to spontaneous rather than iatrogenic causes, as long as retroorbital blood sampling is performed as instructed during training. The role of the postapproval monitoring process becomes essential in this aspect of continuing assurance of laboratory personnel proficiency at this and other clinical skills.

Retroorbital blood collection is a controversial method due to perceived animal welfare concerns, yet our study indicates that instruction through an organized hands-on instructional class prepares trainees adequately to perform the technique with competence. Research personnel will have to evaluate the literature and critically consider the blood collection site that is optimal for their specific studies, considering the advantages and disadvantages of each method and the potential effect of the collection site on the blood parameters being measured. Importantly, accessing the retroorbital route for experimental or clinical purposes remains a valid option when large volumes of blood are needed to achieve study outcomes.

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References

1. **Abatan OI, Welch KB, Nemzek JA.** 2008. Evaluation of saphenous venipuncture and modified tail-clip blood collection in mice. J Am Assoc Lab Anim Sci **47:**8–15.
2. **Ayers JD, Rota PA, Collins ML, Drew CP.** 2012. Alternatives to retroorbital blood collection in hispid cotton rats (*Sigmodon hispidus*). J Am Assoc Lab Anim Sci **51:**239–245.
3. **Bardelmeijer HA, Buckle T, Ouwehand M, Beijnen JH, Schellens JH, van Tellingen O.** 2003. Cannulation of the jugular vein in mice: a method for serial withdrawal of blood samples. Lab Anim **37:**181–187.
4. **Barrios M, Rodriguez-Acosta A, Gil A, Salazar AM, Taylor P, Sánchez EE, Arocha-Piñango CL, Guerrero B.** 2009. Comparative hemostatic parameters in BALB/c, C57BL/6, and C3H/He mice. Thromb Res **124:**338–343.
5. **Boston University Research Compliance Research Committees.** [Internet]. 2014 Blood collection guidelines. [Cited 23 May 2014] Available at: http://www.bu.edu/orccommittees/iacuc/policies-and-guidelines/blood-collection-guidelines/.
6. **Chan YK, Davis PF, Poppitt SD, Sun X, Greenhill NS, Krishnamurthi R, Przepiorski A, McGill AT, Krissansen GW.** 2012. Influence of tail versus cardiac sampling on blood glucose and lipid profiles in mice. Lab Anim **46:**142–147.
7. **Christensen SD, Mikkelsen LF, Fels JJ, Bodvarsdóttir TB, Hansen AK.** 2009. Quality of plasma sampled by different methods for multiple blood sampling in mice. Lab Anim **43:**65–71.
8. **Clifford P, Melfi N, Bogdanske J, Johnson EJ, Kehler J, Baran SW.** 2013. Assessment of proficiency and competency in laboratory animal biomethodologies. J Am Assoc Lab Anim Sci **52:**711–716.
9. **Coman JL, Buck WR, Fan LP, Niquette AL, Strasburg DJ.** 2010. PS6. Suitability of the submandibular blood sampling technique for serial blood sampling in individual Mice. Abstract presented at the 2010 American Association Laboratory Animal Science National Meeting, Atlanta, Georgia. J Am Assoc Lab Anim Sci **49:**656–657.
10. **Cornell University Institutional Animal Care and Use Committee.** [Internet]. 2014. Animal Care and Use Procedure 408: Retro-orbital bleeding. [Cited 23 May2014] Available at: http://www.research.cornell.edu/care/documents/ACUPs/ACUP408.pdf.
11. **Douglass RJ, Kuenzi AJ, Wilson T, Van Horne RC.** 2000. Effects of bleeding nonanesthetized wild rodents on handling mortality and subsequent recapture. J Wildl Dis **36:**700–704.
12. **Duke University Animal Care and Use Program.** [Internet]. 2014. Guidelines for retro-orbital blood collection (in mice). [Cited 23 May2014] Available at: http://vetmed.duhs.duke.edu/GuidelinesforRetroOrbitalBloodCollection.html.
13. **Dürschlag M, Würbel H, Stauffacher M, Von Holst D.** 1996. Repeated blood collection in the laboratory mouse by tail incision—modification of an old technique. Physiol Behav **60:**1565–1568.
14. **Faurie C, Khadra M.** 2012. Technical competence in surgeons. ANZ J Surg **82:**682–690.
15. **Fernández I, Peña A, Del Teso N, Pérez V, Rodriguez-Cuesta J.** 2010. Clinical biochemistry parameters in C57BL/6J mice after blood collection from the submandibular vein and retroorbital plexus. J Am Assoc Lab Anim Sci **49:**202–206.
16. **Forster S, Mead A, Sears M.** 1979. An interophthalmic communicating artery as explanation for the consensual irritative response of the rabbit eye. Invest Ophthalmol Vis Sci **18:**161–165.
17. **Frederick National Lab Animal Care and Use Committee.** [Internet]. 2014. Guidelines for rodent blood collection. [Cited 23 May 2014] Available at: http://ncifrederick.cancer.gov/Lasp/acuc/Frederick/Media/Documents/ACUC20.pdf.
18. **Gitlin L, Benoit L, Song C, Cella M, Gilfillan S, Holtzman MJ, Colonna M.** 2010. Melanoma differentiation-associated gene 5 (MDA5) is involved in the innate immune response to Paramyxoviridae infection in vivo. PLoS Pathog **6:**e1000734.
19. **Golde WT, Gollobin P, Rodriguez LL.** 2005. A rapid, simple, and humane method for submandibular bleeding of mice using a lancet. Lab Anim (NY) **34:**39–43.
20. **Grouzmann E, Cavadas C, Grand D, Moratel M, Aubert JF, Brunner HR, Mazzolai L.** 2003. Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice. Pflugers Archiv **447**:254–258.
21. **Harwerth RS, Crawford ML, Frishman LJ, Viswanathan S, Smith EL 3rd, Carter-Dawson L.** 2002. Visual field defects and neural losses from experimental glaucoma. Prog Retin Eye Res **21:**91–125.
22. **Heimann M, Käsermann HP, Pfister R, Roth DR, Bürki K.** 2009. Blood collection from the sublingual vein in mice and hamsters: a suitable alternative to retrobulbar technique that provides large volumes and minimizes tissue damage. Lab Anim **43:**255–260.

23. **Heimann M, Roth DR, Ledieu D, Pfister R, Classen W.** 2010. Sublingual and submandibular blood collection in mice: a comparison of effects on body weight, food consumption, and tissue damage. Lab Anim **44:**352–358.
24. **Hem A, Smith AJ, Solberg P.** 1998. Saphenous vein puncture for blood sampling of the mouse, rat, hamster, gerbil, guinea pig, ferret, and mink. Lab Anim **32:**364–368.
25. **Hoff J.** 2000. Methods of blood collection in the mouse. Lab Anim (NY) **29:**47–53.
26. **Holmberg H, Kiersgaard MK, Mikkelsen LF, Tranholm M.** 2011. Impact of blood sampling technique on blood quality and animal welfare in haemophilic mice. Lab Anim **45:**114–120.
27. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
28. **Johns Hopkins University Animal Care and Use Committee.** [Internet]. 2014. Retroorbital bleeding of rodents guideline. [Cited 23 May2014] Available at: http://web.jhu.edu/animalcare/procedures/retro-orbital.html.
29. **Kurawattimath V, Pocha K, Mariappan TT, Trivedi RK, Mandlekar S.** 2012. A modified serial blood sampling technique and utility of dried-blood spot technique in estimation of blood concentration: application in mouse pharmacokinetics. Eur J Drug Metab Pharmacokinet **37:**23–30.
30. **Le Net JL, Abbott DP, Mompon RP, Leblanc B.** 1994. Repeated orbital sinus puncture in rats induces damages to optic nerve and retina. Abstract presented at the American Society for Veterinary Clinical Pathology 29th Annual Meeting, Montreal, Quebec, Canada, 30 October – 04 November 1994. Vet Pathol **31:**621.
31. **Li T, Barnett A, Rogers KL, Gianchandani YB.** 2009. A blood sampling microsystem for pharmacokinetic applications: design, fabrication, and initial results. Lab Chip **9:**3495–3503.
32. **Marx JO, Brice AK, Boston RC, Smith AL.** 2013. Incidence rates of spontaneous disease in laboratory mice used at a large biomedical research institution. J Am Assoc Lab Anim Sci **52:**782–791.
33. **Medzhitov** R. 2008. Origin and physiological roles of inflammation. Nature **454:**428–435.
34. **National Centre for the Replacement Refinement & Reduction of Animals in Research.** [Internet]. 2014. Blood sampling: mouse—retroorbital (nonsurgical/terminal). [Cited 23 May2014] Available at: http://www.nc3rs.org.uk/bloodsamplingmicrosite/page.asp?id=386.
35. **National Institutes of Health Office of Animal Care and Use Animal Research Advisory Committee.** [Internet]. 2012. Guidelines for survival bleeding of mice and rats. [Cited 23 May2014] Available at: http://oacu.od.nih.gov/ARAC/documents/Rodent_Bleeding.pdf.
36. **Nemzek JA, Bolgos GL, Williams BA, Remick DG.** 2001. Differences in normal values for murine white blood cell counts and other hematological parameters based on sampling site. Inflamm Res **50**:523–527.
37. **Nugent Britt CC, Blackwell A, Hall C, Story J, Meshaw A, Corniceli J, Rosebury-Smith W, Hollister B.** 2011. The effects of submandivular vein blood collection on the health of female NCr nu/nu, CD1, and CB.17 SCID mice. Abstract presented at the 2011 American Association for Laboratory Animal Science National Meeting, San Diego, California. J Am Assoc Lab Anim Sci **50:**791.
38. **Nyuyki KD, Maloumby R, Reber SO, Neumann ID.** 2012. Comparison of corticosterone responses to acute stressors: chronic jugular vein versus trunk blood samples in mice. Stress **15:**618–626.
39. **Price JE, Barth RF, Johnson CW, Staubus AE.** 1984. Injection of cells and monoclonal antibodies into mice: comparison of tail vein and retroorbital routes. Proc Soc Exp Bio Med **177**:347-353.
40. **Raabe BM, Artwohl JE, Purcell JE, Lovaglio J, Fortman JD.** 2011. Effects of weekly blood collection in C57BL/6 mice. J Am Assoc Lab Anim Sci **50:**680–685.
41. **Rogers IT, Holder DJ, McPherson HE, Acker WR, Brown EG, Washington MV, Motzel SL, Klein HJ.** 1999. Influence of blood collection sites on plasma glucose and insulin concentration in conscious C57BL/6 mice. Contemp Top Lab Anim Sci **38**:25–28.
42. **Sadler AM, Bailey SJ.** 2013. Validation of a refined technique for taking repeated blood samples from juvenile and adult mice. Lab Anim **47:**316–319.
43. **Schnell MA, Hardy C, Hawley M, Propert KJ, Wilson JM.** 2002. Effect of blood collection technique in mice on clinical pathology parameters. Hum Gene Ther **13:**155–161.
44. **Schoch A, Thorey IS, Engert J, Winter G, Emrich T.** 2014. Comparison of the lateral tail vein and the retroorbital venous sinus routes of antibody administration in pharmacokinetic studies. Lab Anim (NY) **43:**95–99.
45. **Sharma A, Fish BL, Moulder JE, Medhora M, Baker JE, Mader M, Cohen EP.** 2014. Safety and blood sample volume and quality of a refined retroorbital bleeding technique in rats using a lateral approach. Lab Anim (NY) **43:**63–66.
46. **Shirasaki Y, Ito Y, Kikuchi M, Imamura Y, Hayashi T.** 2012. Validation studies on blood collection from the jugular vein of conscious mice. J Am Assoc Lab Anim Sci **51:**345–351.
47. **Smith RS, Sundberg JP.** [Internet]. 1995. Inbred C57 black mice: microphthalmia and ocular infections. JAX notes. [Cited 23 May 2014] Available at: http://jaxmice.jax.org/jaxnotes/archive/463a.html.
48. **Steel CD, Stephens AL, Hahto SM, Singletary SJ, Ciavarra RP.** 2008. Comparison of the lateral tail vein and the retroorbital venous sinus as routes of intravenous drug delivery in a transgenic mouse model. Lab Anim (NY) **37:**26–32.
49. **Taylor R, Hayes KE, Toth LA.** 2000. Evaluation of an anesthetic regimen for retroorbital blood collection from mice. Contemp Top Lab Anim Sci **39**:14–17.
50. **Tomlinson R, Abramson M, Edwards K, Reed G, Diehl A, Medrano J.** 2004. Comparing the effectiveness of mandibular versus retroorbital blood collection. Abstracts presented at the American Association for Laboratory Animal Science National Meeting, Tampa Bay, Florida, Contemp Top Lab Anim Sci **23:**53.
51. **Ullman-Culleré MH, Foltz CJ.** 1999. Body condition scoring: a rapid and accurate method for assessing health status in mice. Lab Anim Sci **49:**319–323.
52. **University of California–San Francisco Institutional Animal Care and Use Committee.** [Internet]. 2014. Standard procedure: retroorbital blood collection in mice. [Cited 23 May 2014] Available at: http://www.research.ucsf.edu/aw/Policies/awStandardProcedures.asp.
53. **van Herck H, Baumans V, Brandt CJ, Hesp AP, Sturkenboom JH, van Lith HA, van Tintelen G, Beynen AC.** 1998. Orbital sinus blood sampling in rats as performed by different animal technicians: the influence of technique and expertise. Lab Anim **32:**377–386.
54. **Van Herck H, Baumans V, Van der Craats NR, Hesp AP, Meijer GW, Van Tintelen G, Walvoort HC, Beynen AC.** 1992. Histological changes in the orbital region of rats after orbital puncture. Lab Anim **26:**53–58.
55. **Williams MV, Denekamp J.** 1982. Blood samples from mice: the influence of sampling site. Br J Radiol **55:**782–784.
56. **Yardeni T, Eckhaus M, Morris HD, Huizing M, Hoogstraten-Miller S.** 2011. Retroorbital injections in mice. Lab Anim (NY) **40:**155–160.