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Evaluation of Submental Blood Collection in Mice (*Mus musculus*)

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The submental route is an option for nonterminal and serial blood collection in mice. This study compared the submental route to the maxillary route (also referred to as the submandibular route). The study used male CD1 and C57BL/6 strains of mice in 2 age groups: 8 and 19 wk. To simulate repeated toxicokinetic blood collection, blood was collected from each mouse at 1 and 24-h on Study Day 1, and at 1, 4 and 24 h on Study Day 16. Food consumption, body weights, and clinical observations were assessed daily. No apparent differences were found between the 2 blood collection sites in terms of either food consumption or body weight. Mice bled via the submental route showed fewer adverse clinical effects than did mice bled via the maxillary route. Clinical pathology showed no differences between the 2 methods. In addition, 7 trained technicians, who were inexperienced with the 2 bleeding methods prior to these evaluations, were surveyed to gain insights into expectations and overall experience of using the 2 routes. All 7 technicians preferred the submental route to the maxillary route. Furthermore, the average time needed to become proficient in submental blood collection (1.6 d) was less than that required to become proficient in maxillary blood collection (2.6 d). The qualitative aspects of this study, combined with fewer adverse clinical events, suggest ways to improve both animal and staff welfare. Our findings suggest that the submental route is safe, effective, and easier than the maxillary route for nonterminal serial blood collection in mice.

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Introduction

Nonclinical investigations rely on analyzing blood to assess animal health and to measure levels of test articles and/or their associated metabolites. Nonterminal collections offer the advantage of serial assessment of an individual animal. Blood is commonly used and readily accessible, but for research conducted in mice, blood volume is often a limiting factor. Mice offer many advantages in research due to their small size, short life span, ease of maintenance, and readily available genetic information,[1] but the small size of mice, which facilitates their use in research, provides a limited volume of blood available for analysis. According to the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs), the average mouse has approximately 58.5 mL[8,9] of blood per kg of bodyweight. This translates into a total blood volume of approximately 1.46 mL for a 25-g mouse, but only a fraction of the total blood (no more than 10% of circulating blood volume)[7] can be removed for survival collection.

While serial microsampling[10] may provide adequate amounts of blood for analysis, the optimal blood collection technique to use in mice remains debatable. Some survival collection methods (for example, saphenous vein, tail vein, maxillary, jugular vein, and dorsal pedal vein) do not require anesthesia, which is often preferable to those that do (for example, tail snip and orbital sinus).[14] Several of the survival collection methods (for example, saphenous vein, dorsal pedal vein, and tail vein) are only acceptable for collection of smaller blood volumes (5 to 10 µL), but are not practical when volumes larger than 50 µL are needed. The jugular vein and the orbital sinus may be used to collect larger blood volumes, but these methods present difficulties. Collection from the jugular vein is technically challenging and may require 2 persons,[14] and the orbital sinus route is coming under increased scrutiny with regard to animal welfare[17]. Moreover, the orbital sinus route recommended for serial collection,[11] is highly dependent on the experience and technique of the phlebotomist,[17] and is increasingly being recommended for only terminal sampling.[11]

Two alternative survival collection methods are maxillary (also known as the submandibular) route, which targets the cheek area that includes[15] the superficial temporal, submandibular, linguofacial, and facial veins, and the submental route, which targets the vasculature under the chin. The submandibular, submental, and retroorbital collection methods were recently compared.[15] This study highlighted several advantages of the submental method, such as fewer adverse clinical signs, reduced extraneous loss of blood, no requirement for sedation and the larger high quality nonterminal samples, as compared with submandibular and orbital sinus collection. Our facility rarely uses orbital sinus collection, but frequently employs the maxillary method for nonterminal collections, so we were particularly interested in the advantages described for the submental method.

Before implementing the submental collection method at our organization, we wanted to confirm the previously published results by comparing the maxillary/submandibular and submental methods. In addition to confirming those results, we also wanted to compare the 2 sites in terms of parameters reflecting animal wellbeing (that is, clinical observations, food consumption, body weight) and the difficulty in becoming proficient in each technique. We also wanted to compare the general perception of the 2 methods among the technical staff. To this end, we chose to compare the 2 methods using male mice of different ages and strains.

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Materials and Methods

Animals. Male CD1 and C57BL/6 mice (8 and 19 wk old) were obtained from Charles River Labs (Wilmington, MA). Mice weighed between 21.4 g and 53.5 g. The mice were individually housed and assigned to study groups of 5 mice per group/strain (submental and maxillary) without randomization after acclimation. Mice were cared for in accordance with the *Guide for the Care and Use of Laboratory Animals* at an AAALAC-accredited facility. They were housed in Allentown polycarbonate cages on ASPEN-CHIP bedding (Nepco, Warrensburg, NY), and enrichment items (Enviro-Dry ™ and a polycarbonate tunnel) were provided per facility standard operating procedures. The study protocol, 999-953-02, was approved by the Institutional Animal Care and Use Committee (IACUC). The mice had unlimited access to Block Lab Diet (Certified Rodent Diet #5002, PMI Nutrition International) and tap water via an automatic water system. The housing room had 12-h alternating light and dark cycles and was maintained between 68 to 79 °F (20 to 26 °C) and between 30 to 70% humidity. The animals were assigned to 8 groups based on age and strain (Table 1).

Technician training. Several weeks before the study began, 7 technicians who were unfamiliar with the maxillary and submental collection methods were chosen to participate in training on both blood collection methods. More technicians were selected for training than were needed for study completion to ensure that an adequate number of trained technicians were available for both techniques. These technicians had an employment tenure of 1 to 5 y. Trainee requirements were to have no prior exposure to maxillary or submental blood collection in mice, to be proficient in mouse handling, and to be proficient with another venous blood collection method in rats. These requirements permitted them to be familiar with mouse handling and the general concept of blood collection, but not to be biased when comparing the maxillary and submental collection methods.

Technicians were assigned randomly to an initial training group and collection route, but eventually received training in both collection routes. They focused learning to use on one collection route at a time, received 2 h per day of training for 3 consecutive days, and then tested their proficiency twice daily as needed for the next 2 d. Attempts were discontinued if proficiency was not achieved after 4 attempts. Once technicians completed training on their first route, they received training for the other route using the same schedule as described above. Technicians were deemed proficient with a route when they could fulfill the following requirements: collect blood from 12 animals in a defined timeframe, of which 6 samples must be from each side (left or right) of the animals respectively, with a maximum of 2 collection attempts per animal, and no clotted or insufficient samples.

Table 1. Experimental Study Design

Group (n = 5)	Age (wk)	Mouse Strain	Collection Route
1	8	CD	maxillary
2	8	CD	submental
3	19	CD	maxillary
4	19	CD	submental
5	8	C57BL/6	maxillary
6	8	C57BL/6	submental
7	19	C57BL/6	maxillary
8	19	C57BL/6	submental

Study. To simulate a pharmacokinetic study design involving repeated blood collections for bioanalytical analysis, a theoretical 'dose' time of 1000 AM was chosen on a day called Study Day 1. However, mice were not dosed. Blood was collected from all mice on Study Days 1 (1 and 24 h) and 16 (1, 4 and 24 h), for a total of 5 collections.

Blood collection. Blood collection volumes were determined based on recent body weights of the mouse.[7] Equipment included 5 mm Lancets (Goldenrod Animal Lancet, MEDIPOINT, Mineola, NY), Greiner Bio-One MiniCollect 0.5 mL K_2EDTA blood collection tubes, and Medline Non-Woven Gauze Sponges 4 × 4 in. (10.16 × 10.16 CM). On Day 1 of the study, at least 0.1 mL of blood was collected at the 1-h and 24-h timepoints for each mouse. On Day 16, at least 0.1 mL of blood was collected at the 1-h, 4-h, and 24-h timepoints. Mice were euthanized using an IACUC-approved method and necropsied after the 24-h blood collection timepoint on Day 16.

During the study, technicians were divided into 2 groups for collection via maxillary and submental methods based on their proficiency with each method. Each technician was permitted a maximum of 3 collection attempts per mouse per time point. If a sample was not obtained, a second trained technician would attempt a collection (maximum of 3 attempts). If blood still could not be collected, the mouse was placed back in its cage until the next time point. Technicians were permitted to choose the side on which to bleed (right or left side of the animal); however, if their first attempt was unsuccessful, they were required to switch sides for the second attempt. Mice were conscious during collections (maxillary and submental).

The submental method was performed as follows. The mouse was restrained by grasping the skin at the nape of the neck, along the cervical and thoracic region between the thumb and index finger, referred to as a 'scruff hold.'[12] The grip tension was sufficient to tilt the head back and limit movement, while exposing the submental/chin region, but loose enough to not restrict the airway. Regardless of visibility, veins could be located by anatomic landmarks, which consisted of a whorl of hair just ventral to the cervical region.

For CD1 mice, the inferior labial and facial veins[2] were used for the submental blood collection; these veins are located halfway between the ventral hair whorl and corner of the mouth (Figure 1A). The inferior labial vein runs along the dorsal border of the body of the mandible, parallel to the ventral edge of the buccinator muscle. The facial vein courses along the ventral aspect of the masseter muscle and crosses the body of the mandible along the rostral edge of this muscle.[2] For the C57BL/6 mice, the targeted vessels are located approximately 2 mm anterior (cranial) and 1 to 2 mm lateral to the hair whorl (Figure 1B).

After the vein was punctured with a 5-mm lancet, the mouse was held in a prone position to permit blood to drip freely into the collection tube until the desired volume was obtained. Once collection was complete, the mouse was released from the scruff hold and any residual blood was removed with gauze. Mice were returned to the home cage after confirmation that bleeding had stopped. If bleeding did not stop immediately after release of the restraint hold, compression was applied to the collection site with gauze, as necessary.

The maxillary method was performed by restraining the mouse in a manner similar to that used for the submental method; however, a tighter grip of the scruff was necessary to ensure that the skin around the cheeks was taut but did not restrict breathing. The superficial temporal, submandibular, linguofacial, and facial veins, which are accessed using this

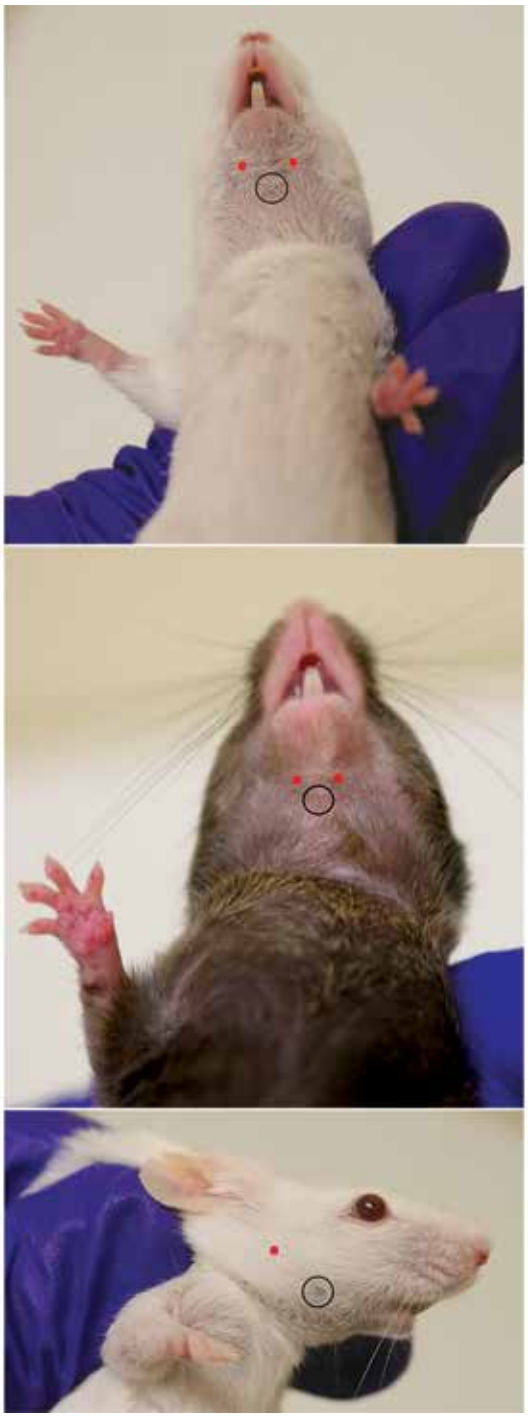

Figure 1. Landmarks for submental blood collection in CD1 mice. A) The inferior labial and facial veins, which are the vessels used for submental blood collection (red filled circles), are located halfway between the ventral hair whorl (black open circle) and corner of the mouth. The inferior labial vein runs along the dorsal border of the body of the mandible, parallel to the ventral edge of the buccinator muscle. The facial vein courses along the ventral aspect of the masseter muscle and crosses the body of the mandible along the rostral edge of this muscle. B) The targeted vessels (red filled circles) are located approximately 2 mm anterior and 1–2 mm lateral to the hair whorl (black open circle). These are slightly smaller distances as compared with those for CD1 mice. C) Maxillary blood collection targets the cheek area that includes the superficial temporal, submandibular, linguofacial, and facial veins. A dimple (open circle) is visible near the jawline. From the dimple, the vasculature (red filled circle) is located approximately 3 mm diagonally toward the ear on CD1 mice and approximately 1–2 mm diagonally toward the ear on the C57BL/6 mice.

collection method, are not visible and must be located using physical landmarks. When the mouse is held correctly, a visible dimple appears near the jawline (Figure 1C). From the dimple, the vasculature is located approximately 3 mm diagonally toward the ear on CD1 mice and approximately 1 to 2 mm diagonally toward the ear on the C57BL/6 mice. After accessing the vein with a 5-mm lancet, the mouse was held in a lateral position over the collection tube, and the blood was allowed to drip freely until the desired volume was collected. Once the collection was complete, the hold on the mouse was loosened slightly, and gauze was applied with pressure to the collection site until bleeding stopped. The mouse was then returned to its home cage. Any difficulties or observations were recorded at the time of occurrence. The number of attempts at each collection were recorded.

Detailed clinical observations. Qualitative clinical observations were performed once daily by trained technicians. Observations included but were not limited to evaluation of the skin, fur, eyes, ears, nose, oral cavity, thorax, abdomen, external genitalia, limbs and feet, respiratory and circulatory effects, autonomic effects such as salivation, nervous system effects including tremors, convulsions, reactivity to handling, and unusual behavior. The blood collection site was carefully examined, and the hydration status was documented.

Observations after blood collection were recorded by various study technicians depending on scheduling for the day. Previous clinical observations were visible to the technician at the time of assessment. Observations that warranted additional veterinary intervention were submitted to the veterinary staff for further evaluation.

Body weight and food consumption. Mice were weighed daily on a calibrated scale. Daily weights were compared with the previously collected weights to determine the effects of blood collection on each mouse.

Food consumption was quantitatively measured daily based on remaining weight of food. To prevent dehydration, mice received Hydrogel (ClearH_2O, Portland, ME) for 24 h before each blood collection.

Clinical pathology. Blood samples were collected in Greiner Bio-One MiniCollect 0.5-mL K_2EDTA tubes and visually inspected for clotting. A small aliquot (approximately 40 to 50 µL) of blood was transferred to a standard capillary tube and one end was capped with a clay sealant (Critoseal). Capillary tubes were centrifuged at 10,000 × *g* at ambient temperature for 5 min. A manual hematocrit evaluation was performed using a microhematocrit reading device (EZ Reader). All remaining blood from each sample was processed to plasma, visually inspected for hemolysis, and evaluated for glucose and total protein content using an automated chemistry analyzer (Beckman Coulter AU5800). Clotted samples were not evaluated. Any residual sample was discarded after analysis.

Necropsy. After the final blood collection on Day 17, all mice received a limited gross necropsy of the head and neck. Mice were euthanized via an IACUC-approved method (CO_2 inhalation followed by exsanguination). The head, neck, and mouth of each mouse were examined internally and externally for any sign of macroscopic hemorrhage and/or bruising.

Statistical Analysis. We fit a 4-factor split-plot ANOVA, with whole plot factors of strain, age and technique, and a repeated measure of time using PROC GLIMMIX of SAS (version 9.4; SAS Institute, Cary, NC). The optimum covariance structure was selected from Cholesky-root (CHOL), compound-symmetry (CS), heterogeneous compound-symmetry (CSH), spatial power (sp(pow)), and first-order heterogeneous autoregressive

(ARH(1)) covariance structures by choosing the model with the smallest corrected AIC (AICc). Optimum covariance structure was determined to be CHOL for the glucose endpoint, CS for total protein, ARH(1) for daily food consumption, and CHOL for body weight.

Results

Detailed clinical observations. Clinical observations noted for the submental collection method included decreased activity and dehydration on Days 1, 2, 16, or 17 after blood collection (Table 2). For the maxillary method, clinical observations included decreased activity, shallow breathing, convulsions, lateral recumbency, dehydration, hunched posture, and twitching on Days 1, 2, 16, or 17 after blood collection (Table 3). The submental route had fewer clinical observations than did the maxillary route (8 compared with 47, respectively). More clinical observations were made for the 19-wk-old mice (groups 3 and 7) bled via the maxillary route regardless of the mouse strain. The number of attempts or the technician who collected the blood did not affect on the observed clinical signs. One mouse from Group 5 (C57BL/6, 8-wk age, maxillary) was found with reduced activity, hunched posture, severe dehydration, shallow breathing, hypothermia and piloerection on Day 5. Due to poor prognosis, this mouse was euthanized.

Food consumption and body weight. For mean body weights, the percent weight change from Days 1 to 16 ranged from −4.1% to +5.7% for mice bled via the submental method and from −4.3% to +4.5% for mice bled via the maxillary method (Table 4). Within each group, the mean food consumption was the same on Days 1 and 16 (Table 5).

Clinical pathology. Blood samples were collected from all mice as planned except for 3. Day 16 samples could not be collected from a group 5 (C57BL/6, 8 wk age, maxillary) mouse that was euthanized on Day 5. Samples were not collected from one mouse each at 1 h (C57BL/6, 8 wk age, submental) on Day 1 and 24 h (C57BL/6, 19 wk age, maxillary) on Day 2, due to reaching the highest permitted number of attempts.

No samples from either method exhibited hemolysis, and only one sample from each method was clotted. Four samples from the submental method (group 6), and one sample from the maxillary method (group 3) had less than the desired volume. One sample intended for collection via the submental method was not collected due to lethargy noted in the mouse (group 6), and one sample intended for the maxillary method of collection (group 7) was not collected due to reaching the maximum number of allowed attempts . Mean hematocrits and

Table 2. Clinical observations noted with submental route of collection

Clinical sign	CD-1 8 Wk Submental	CD-1 19 Wk Submental	C57BL/6 8 Wk Submental	C57BL/6 19 Wk Submental	Total No. of observations
Decreased activity	0 (0)[a]	0 (0)	2 (2)	3 (2)	5
Dehydration	1 (1)	0 (0)	2 (1)	0 (0)	3

[a]The first number describes the total number of times the observation was made, and the number in parentheses indicates the total number of animals with the indicated observation.
No. = Number

Table 3. Clinical Observations noted with maxillary route of collection.

Clinical sign	CD-1 8 Wk Maxillary	CD-1 19 Wk Maxillary	C57BL/6 8 Wk Maxillary	C57BL/6 19 Wk Maxillary	Total No. of observations
Decreased activity	0 (0)[a]	6 (4)	5 (2)	4 (4)	15
Shallow breathing	0 (0)	3 (2)	1 (1)	1 (1)	5
Dehydration	0 (0)	0 (0)	4 (2)	5 (1)	9
Hunched posture	0 (0)	0 (0)	2 (1)	7 (1)	9
Clonic convulsions	0 (0)	1 (1)	0 (0)	1 (1)	2
Tonic convulsions	0 (0)	2 (1)	0 (0)	1 (1)	3
Lateral recumbency	0 (0)	2 (2)	0 (0)	0 (0)	2
Twitching	0 (0)	2 (2)	0 (0)	0 (0)	2

[a]The first number describes the total number of times the observation was made, and the number in parentheses indicates the total number of animals with the indicated observation.
No. = Number

Table 4. Mean body weight values

	CD-1 8 Wk Maxillary (N = 5)	CD-1 8 Wk Submental (N = 5)	CD-1 19 Wk Maxillary (N = 5)	CD-1 19 Wk Submental (N = 5)	C57BL/6 8 Wk Maxillary (N = 5)	C57BL/6 8 Wk Submental (N = 5)	C57BL/6 19 Wk Maxillary (N = 5)	C57BL/6 19 Wk Submental (N = 5)
BW Day -1 (g)	36 (2.1)	35 (1.7)	47 (4.9)	49 (2.9)	22 (0.2)	23 (0.8)	27 (2.7)	29 (3.1)
BW Day 16 (g)	35 (1.1)	37 (1.8)	45 (4.9)	47 (2.1)	23 (0.6)	24 (1.7)	26 (3.2)	28 (2.3)
Percent Change Day 1-16	-2.8%	+5.7%	-4.3%	-4.1%	+4.5%	+4.3%	-3.7%	-3.4%

BW = Body weight; g = grams; the values in parentheses represent the Standard Deviation, SD.

Table 5. Mean Food Consumption (FC) Values

	CD-1 8 Wk Maxillary	CD-1 8 Wk Submental	CD-1 19 Wk Maxillary	CD-1 19 Wk Submental	C57BL/6 8 Wk Maxillary	C57BL/6 8 Wk Submental	C57BL/6 19 Wk Maxillary	C57BL/6 19 Wk Submental
FC Day 1(g)	3	5	2	3	2	2	1.5	2
FC Day 16[a](g)	3	5	2	3	2	2	1.5	2

FC = Food consumption; g = grams
[a]On Day 16, animals were bled 3 times (1, 4 and 24 h) whereas on Day 1 animals were bled twice (1 and 24 h)

Table 6. Mean Clinical Pathology parameters

Day and Time Point	CD 1 8 Wk Maxillary	CD 1 8 Wk Submental	CD 1 19 Wk Maxillary	CD 1 19 Wk Submental	C57BL/6 8 Wk Maxillary	C57BL/6 8 Wk Submental	C57BL/6 19 Wk Maxillary	C57BL/6 19 Wk Submental
Hematocrit (%)[a]								
Day 1 (1 h)	48.0	47.6	46.4	48.2	51.2	51.3	48.6	49.8
Day 1 (24 h)	38.0	39.0	37.0	36.8	40.2	44.0	39.5	40.8
Day 16 (1 h)	49.4	49.4	48.2	48.2	52.0	51.8	49.3	49.8
Day 16 (4 h)	42.0	39.4	42.0	40.6	42.8	42.0	41.0	39.5
Day 16 (24 h)	34.6	35.4	35.8	34.4	35.0	34.8	35.6	34.6
Total Protein (g/dL)[a]								
Day 1 (1 h)	5.5	5.3	5.4	5.5	5.1	5.1	5.0	5.3
Day 1 (24 h)	5.3	5.1	5.4	5.3	4.7	4.9	5.0	5.1
Day 16 (1 h)	5.7	5.7	5.8	5.9	5.2	5.3	5.3	5.4
Day 16 (4 h)	5.2	5.1	5.3	5.3	4.7	4.6	4.7	4.7
Day 16 (24 h)	5.3	5.4	5.4	5.5	5.0	5.0	5.2	5.1
Glucose (mg/dL)[a]								
Day 1 (1 h)	200	200	177	178	205	235	194	188
Day 1 (24 h)	194	215	201	196	231	195	184	187
Day 16 (1 h)	198	186	206	174	229	204	174	193
Day 16 (4 h)	199	199	198	199	207	228	190	220
Day 16 (24 h)	216	200	186	203	185	206	174	205

[a]The Standard Deviation for hematocrit ranged from 2 to 5, Total protein ranged from 0.1 to 0.4 and glucose ranged from 11 to 37 in the 8 groups.

total protein were comparable across ages, strains, and blood collection methods at each time point. (Table 6). Mean glucose values were within the historic reference values for our site, with no biologically meaningful differences between blood collection methods.

Necropsy. Lesions seen during the gross necropsies were similar regardless of age, strain, and collection site. Lesions seen at both sites included red foci and discoloration at the venipuncture site.

Blood collection training and feedback. During the training period before the start of the study, 4 and 3 of 7 technicians respectively gained proficiency with the submental and maxillary sites. One technician became proficient for both routes, and one technician could not obtain proficiency for either route. Technicians who became proficient at the submental route required 1 to 2 days less time to gain proficiency than did those who became proficient at the maxillary route (Table 7).

All participating technicians generally preferred the submental collection method over the maxillary route. They stated that the submental collection route contributed to an enhanced culture of care due to 1) fewer complications for the mice, 2) reduced need for firm restraint and thus less stress on the mouse and 3) ergonomics that did not require abnormal twisting or contorting of the wrist as was required for the maxillary method.

Statistical analysis. The 4-way interaction between strain, age, technique, and time was the highest order significant effect for glucose, total protein, and body weight. Post-hoc comparisons were made between submental and maxillary blood drawing techniques within each unique combination of strain, age, and timepoint, however, no post-hoc comparisons were found to be significant. For the remaining endpoint, food mean daily consumption (FDC), the three-way interaction between strain, age, and technique was found to be the highest order significant effect. Post-hoc comparisons were made between techniques for each unique combination of strain and age, but once again, none were found to be significant.

Discussion

A previous study described the superiority of the submental blood collection method as compared with retroorbital and submandibular/maxillary collection methods in mice.[15] That study compared body weight, sample quality (based on hemolysis and clotting), ease of collection, and mouse trauma across the 3 collection methods. For nonterminal blood collections requiring 100-µL blood volumes, the maxillary method has been used at our facility. Like the previous authors,[15] we had difficulty in training our technical staff in the maxillary method and consistently collecting samples of sufficient quality for analysis. The maxillary technique has been associated with mouse mortality, and many phlebotomists find that the necessary restraint is difficult to perform. In addition, the location of the lancet stick in the cheek region has led to concern over potential animal stress, and thus, a general dislike of maxillary collections.

Table 7. Technician training and proficiency

Technician Identification	Submental-Proficiency Attempts	Submental-Received Proficiency	Maxillary- Proficiency Attempts	Maxillary- Received Proficiency
Tech 1	1	Yes, Day 4	1	Yes, Day 4
Tech 2	4	Not achieved	2	Yes, Day 5
Tech 3	4	Not achieved	1	Yes, day 4
Tech 4	1	Yes, Day 5	4	Not achieved
Tech 5	2	Not achieved	2	Not achieved
Tech 6	2	Yes, Day 3	4	Not achieved
Tech 7	2	Yes, Day 3	4	Not achieved

Our training and technical staff are eager to learn and/or develop improved study methods and welcomed the opportunity to investigate the potential advantages of submental collection. In addition to confirming the previously published results,[15] we wanted to compare the maxillary and submental methods for training proficiency, clinical effects on mouse wellbeing, and the general perception of the 2 methods among the technical staff. In addition, we wanted to compare the 2 methods using mice of different ages/sizes and strains. The previous study[15] indicated that one of the advantages of the submental method is the ability to visualize the blood vessels, which is true in albino animals, such as CD1 mice; however, many studies use pigmented strains (for example, C57BL/6). We questioned whether the submental method would still be superior with pigmented mice in which vessel visualization might not be possible.

Our study evaluated only male mice. Male mice have greater muscle mass than females,[4] which could make males the more difficult of the 2 sexes with regard to accessing blood vessels.

We found the submental method to be superior to the maxillary method for both trainability and animal welfare. Although our study was relatively small and used just a few phlebotomists, more individuals were able to demonstrate proficiency with submental collection (4 out of 7) compared with maxillary collection (3 out of 7). Moreover, technicians took less time to achieve proficiency using the submental method compared with the maxillary method. The shorter training period was attributed to familiarity with the submental hold (a standard oral gavage hold, which is more ergonomic than the maxillary hold), more easily identifiable landmarks, and greater comfort with the submental method due to its targeting the area under the chin, which is more distant from the face and mouth of the mouse as compared with the maxillary method. This site was perceived as being less stressful to the mice.

Mice bled using the submental method had fewer clinical observations (that is, occasions of requiring veterinary monitoring and/or treatment) than those bled using the maxillary method. The only 2 noteworthy clinical observations seen with the submental method were reduced activity and loss of skin elasticity. Mice bled using the maxillary method showed these clinical signs as well as occasions of shallow breathing, convulsions, lateral recumbency, hunched posture and twitching. Several factors could account for differences in number of clinical observations, but we believe the cause may be greater increased stress that resulted from the location and firmness of hold required for the maxillary route as compared with the submental route. Future evaluations may better identify causes and determine whether technician experience lessens these occurrences.

Over the 16 d of the study, mice bled using the submental method had a 1.9% increase in mean body weight as compared with a 3.3% decrease in mean body weight in mice bled by the maxillary method. In the absence of concurrent controls, the importance of these body weight changes could not be determined. We hypothesize that contributing causes for body-weight loss could be related to stress and factors such as loss of appetite and reduced eating. The submental method resulted in less bruising and extraneous blood loss due to the cessation of bleeding upon release of the hold, whereas the maxillary collection route often resulted in more bruising and blood loss.

The previously published study[15] found that the overall number of good-quality samples (defined by appropriate volume with limited hemolysis and clotting) was higher for the submental method than the maxillary method. In contrast, our study found that the collection method had no effect on sample quality, as determined by the number of insufficient or missing samples, hematology parameters (hematocrit, hemolysis, and clot formation), and clinical chemistry parameters (total protein and glucose). The levels of hemolysis and clotting were comparable between methods, with no hemolyzed samples and only one sample from each method being clotted. Intended sample volumes were collected with both methods. Two samples were inadequate using the submental method, and one using the maxillary method. Only one was missed from the submental mice, which was not collected due to mouse lethargy, and no samples were missed the maxillary method.

Decreases in hematocrit and total protein were comparable across ages, strains, and blood collection routes. These decreases were expected and were attributed to blood loss associated with the serial collections. Glucose values were within our historic reference range and were not different between collection methods.

The previous study[15] found that the submental method could frequently be performed with a smaller lancet than for the maxillary method. In our study, we used the same lancet size (5 mm) for both collection methods. In preparing for this study, we found that smaller lancets (for example, 4 mm) frequently required multiple attempts to collect the needed blood volume.

In a further examination of the overall effect of the collection method on the mice, we conducted a limited gross necropsy of the blood collection areas (that is, the head and neck) to determine the extent of physical trauma caused by each collection method. Tissue damage was similar across ages, strains, and collection sites. The primary findings were red foci and red discoloration at the venipuncture site.

Many mouse toxicology studies collect blood is collected by cardiac puncture, making each collection a terminal procedure. To establish a toxicokinetic drug profile in blood, blood samples are frequently collected at 6 intervals on Day 1 and on the day before necropsy. Such studies require a minimum of 3 mice per sex per time point to test statistical significance, which requires in a total of 36 mice per sex and group on 2 collection days (3 animals/sex/group/timepoint × 6 time points × 2 collection days).[3] In the present study, the submental route provided

5 serial samples from each mouse on 2 collection days, thereby supporting the goal of reducing the total number of mice required for a typical toxicokinetic study and helping to fulfill the reduction R of the 3 "Rs" of animal welfare.

As discussions and concern grow with regard to creating an overall culture of care in animal research, so do the efforts to improve animal and technician welfare and wellbeing, respectively. Blood collections are an essential component of most clinical and nonclinical studies. Yet despite the common use of needles for blood collection, 'needle phobia' is frequent and is considered to be a medical condition affecting at least 10% of the population.[5,6] Although needle phobia may be expected to occur with the subject receiving an injection or giving a blood sample, empathy of laboratory personnel with the test subject is also an important consideration. Reducing the trauma associated with blood collections is one means of instilling a culture of care. As explained by others,[13,16] "Culture of Care" can be defined in many ways, but one definition includes an organization's effort to achieve improved animal and staff care and welfare, scientific quality, and organizational transparency. As discussed above, our technical staff greatly preferred the submental collection method over the maxillary method, suggesting that use of the submental method could contribute to improved technician wellbeing as compared with the maxillary method. This improvement is attributed to fewer animal welfare issues, a less firm restraint that causes less animal stress, and a more ergonomic hold that does not require twisting or contorting the wrist, as is required for the maxillary method.

Overall, our current study confirmed the previous report.[15] In every respect, the submental method was equal or superior to the maxillary method. As compared with the maxillary route, study technicians found the submental method easier to learn, faster to achieve proficiency, and likely to contribute to an overall improvement in the culture of care. In addition, the method was easily adaptable to different mouse sizes and strains and was associated with fewer welfare concerns than was the maxillary method. As a result, the submental collection is being widely implemented at our site and throughout Charles River Laboratories.

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