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Improving the Quality of EDTA-treated Blood Specimens from Mice

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Nonterminal blood sampling in laboratory mice is a very common procedure. With the goal of improving animal welfare, different sampling sites and methods have been compared but have not achieved a consensus. Moreover, most of these studies overlooked the quality of blood specimens collected. The main preanalytical concern with EDTA-treated blood specimens for hematology analyses is platelet aggregation, which is known to cause analytical errors. Our objective was to find a nonterminal blood sampling method with minimal adverse effects on mice and few or no platelet aggregates. We tested and compared 2 collection sites, 4 sampling methods, and 3 antithrombotic drugs in 80 C57BL6/j male and female mice by evaluating platelet aggregates on blood smears and platelet, WBC, and RBC counts. In addition, the blood collection process was carefully evaluated, and adverse effects were recorded. Platelet aggregation was lower in specimens collected from the jugular vein than from the facial vein, with no effect of the sampling device or the presence of an antithrombotic additive. Highly aggregated specimens were significantly associated with lower platelet counts, whereas aggregation had no effect on WBC or RBC counts. Adverse events during sampling were significantly associated with more numerous platelet aggregates. The jugular vein is thus a satisfactory sampling site in mice in terms of both animal welfare and low platelet aggregation. Using antithrombotic agents appears to be unnecessary, whereas improving sampling conditions remains a key requirement to ensure the quality of EDTA-treated blood specimens from mice.

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Industrial and academic research often require hematology analyses of mouse blood. Consequently, many terminal and nonterminal techniques have become available for blood sampling in mice.[12,21,27,40,42,53] Preanalytical variation in clinical pathology is known to be a major issue.[5,45,49] Although the effects of the blood sampling method on animal welfare have been the subject of many preanalytical hematology and biochemical analyses,[1,6,8,9,15,16,18,24-26,36,47,50-52,54] no agreement has been reached regarding the optimal method for nonterminal blood collection in mice and, to our knowledge, only a few investigations[1,8,15,16,18] have addressed the quality of the resulting blood specimens.

Our own experience of hematology measurements from nonterminal mouse EDTA-blood specimens is that some specimens show both visible clots and platelet aggregation, the latter being detected only from microscopic examination of blood smears.[33] Whereas specimens with visible clots can be eliminated, microscopic platelet aggregates can also interfere with hematology analyses or cause analytical errors, as has been reported in other species including cats.[13,22,31,39] These abnormalities require repeat sampling when possible; otherwise, the number of validated results is decreased. EDTA-treated mouse blood is especially prone to platelet aggregation and clotting.[14,28,43] This characteristic leads to errors in platelet counts (pseudothrombocytopenia) and possible misidentification of platelet aggregates as eosinophils, resulting in false leukocytosis and eosinophilia.[14] In vitro platelet aggregation in mice is due to high platelet counts[34,43] and is influenced by numerous preanalytical factors including the sampling method, collection site, specimen processing, anticoagulant used, the blood:anticoagulant ratio, the mouse strain and genetic alterations.[19,28,30,43] The literature on the influence of preanalytical factors on the quality of CBC analyses in mice is scant,[43] and no agreement has yet been reached regarding the optimal method for nonterminal blood collection in mice. In humans and various animal species, platelet aggregation can be reduced by adding platelet aggregation inhibitors that act at different steps of aggregation. To our knowledge, the addition of such inhibitors to mouse whole blood has not been tested as a means to improve the quality of mice EDTA-treated blood specimens.

The aim of this study was therefore to identify the best preanalytical conditions for nonterminal blood collection in mice, based on animal welfare, scores of platelet aggregation, and platelet, RBC, and WBC counts. The hypotheses we tested were that 1) adding an antithrombotic drug (or multiple such drugs) to the EDTA-treated blood specimen would prevent or at least significantly lower platelet aggregation, 2) the site and the method of collection influence in vitro platelet aggregation, and 3) high-quality blood sampling is a key to reducing platelet aggregation in blood specimens.

Materials and Methods

Experimental design. The criteria used to estimate the quality of EDTA blood specimens were the number of platelet aggregates seen on the blood smears and the results of total platelet, RBC, and WBC counts. The quality of blood specimens was tested in 3 successive steps: (1) adding platelet aggregation inhibitor to the sampling devices, (2) testing different methods of blood collection, and (3) evaluating a combination of

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the most efficient platelet aggregation inhibitor and the most efficient sampling procedure determined in the 2 previous steps. Because judging the quality of each sampling protocol is subjective, quality was scored by an investigator who did not perform the sampling.

Ethical considerations. The experimental protocol was designed according to the European Recommendations for Laboratory Animal Welfare and Protection (Directive 2010/63/UE) and the Institutional Animal Care and User Ethical Committee No. 115 of the National Veterinary School of Toulouse, France (APAFIS no. 26040–202102041206831 v2). Final approval was given by the French Ministry of Higher Education, Research, and Innovation.

Animals. Male (n = 40) and female (n = 40) C57BL/6JRj mice (age, 6 wk) were purchased from Janvier Labs (Saint Berthevin, France). Their microbiologic status was specific pathogen-free (SPF) as defined in the Federation of European Laboratory Animal Science Associations (FELASA) recommendations. All 80 mice were enrolled in the 3 consecutive studies, which were separated by a 6-wk recovery period. Upon receipt in the animal facility, mice were allowed to acclimate for at least 7 d before any experimental procedure. The mice were observed daily by a veterinarian to assess their health and wellbeing and for any signs of pain or discomfort. Groups of 5 same-sex mice were housed in each polycarbonate cage with vegetable corn cob bedding (GM12, Eurocob, Maubourguet, France) in positive-pressure ventilated racks with an airflow of 75 air changes per hour (GM500 IVC Sealsafe Plus Rack, Tecniplast, Lyon, France) with controlled light, temperature, and air renewal. Environmental conditions were a 12:12-h light:dark cycle, ambient temperature of 21± 3 °C, and an average humidity of 46%. All the mice received a complete maintenance diet (S9955-S410, Sniff Specialty Diets, Soest, Germany) and municipal drinking water ad libitum. Enrichments such as polycarbonate tubes, cellulose shelters, and nesting materials (SerLab, London, United Kingdom) were provided in all cages.

Sampling procedures. Before sampling, the mice were randomly selected in terms of sex and experimental variables by drawing pieces of paper previously printed with these items. The mouse selected was transferred from its cage to an induction chamber (Compact Anesthesia Unit for Rodents, Minerve, Esternay, France) that contained 3% isoflurane (Isoflo, Zoetis, Malakoff, France) and 1.2 L/min ambient air flow. The mouse remained there for 2 to 3 min until loss of consciousness. Once the righting reflex was lost, mice were removed from the chamber and either immediately sampled from the facial vein or maintained under anesthesia for jugular vein sampling. Anesthesia was maintained with 2% isoflurane and 200 mL/min ambient air flow through a mouse mask with optical presence detector and coaxial suction (Minerve). During anesthesia maintenance, the mouse was placed on its back on a heating plate included in the anesthesia unit and autoregulated at 36 °C to limit the decrease in body temperature.

Facial vein sampling was performed with a 5-mm single-use lancet (Goldenrod Animal Lancet, Medipoint, Mineola, NY): while the unconscious mouse was held by the scruff of the neck, a puncture was made on the side of the face, at the back of the jaw, by using landmarks previously described.[16,21,25] The dripping blood was then collected directly from the puncture site, which then was compressed for a maximum of 15 s to prevent hematoma formation. For jugular vein phlebotomy, a sufficient level of narcosis was indicated by the absence of animal movement associated with a negative withdrawal reflex and thoracoabdominal breathing. A 1-mL syringe with a 25-gauge needle (25 gauge × 1 in. BD Microlance 3, Becton Dickinson, Franklin Lakes, NJ) was inserted into the jugular vein in a caudocephalic direction and lateral to the sternoclavicular junction, as previously described.[27,50] The anesthetic mask maintained the mouse's head in an extended position suitable for jugular vein access without additional restraint.

After blood collection, mice were placed in a transparent box so that recovery could be monitored closely. Once the mice had completely recovered from anesthesia, they were returned to their home cage.

Study 1: Comparison of 3 antithrombotic drugs. Specimens of blood (200 µL) were collected from the right facial vein of 4 groups of 10 male and 10 female mice by using single-use safety lancets and 200 µL K3-EDTA Minivette POCT (Sarstedt, Nümbrecht, Germany). After checking that the filling of the Minivette was not impaired by the introduction of a 2-µL volume of liquid, 2 µL of iloprost (Ilomedin ND 100 µg/mL, Bayer Healthcare SAS, Loos, France), cangrelor (Kengrexal 50 mg, Novartis, Rueil Malmaison, France), aspirin (Aspegic 1000 mg/5 mL, Sanofi, Paris, France) or 0.9% NaCl (control) was introduced into the Minivette to obtain final concentrations of 3 µmol/mL, 140 µmol/L, and 1 mmol/L for iloprost, cangrelor, and aspirin, respectively. The investigator who performed the sampling was blind to the antithrombotic drug in the Minivette.

Study 2: Comparison of 4 blood sampling procedures. Unsupplemented K3-EDTA specimens were obtained from 4 groups of 10 male and 10 female mice: 3 groups were sampled from the facial vein by using (1) the Minivette (as described in study 1), (2) the Microvette (200 µL K3-EDTA Microvette, Sarstedt, Nümbrecht, Germany), with its capillary placed end-to-end under the blood flow, and (3) by simply collecting drops of blood in an EDTA-treated tube. Blood from the fourth group was sampled from the left jugular vein by using a 25-gauge needle and a 1-mL syringe coated with EDTA solution to ensure rapid aspiration and emptying of the syringe.

Study 3: Effect of combining an antithrombotic drug with the selected sampling procedure. Specimens were obtained from the right jugular vein as described in study 2, from 2 groups of 38 male and 38 female, with the addition of either 2 µL of NaCl or iloprost. After study 3 was completed, the mice were kept in the animal husbandry unit for 1 wk for follow-up and evaluation of the possible consequences of blood removal.

Quality of blood specimen collection. The blood collection process was evaluated by an observer who was not the sampler and was scored as 0 for 'unremarkable' or 1 for 'problematic'. An unremarkable collection was one in which sufficient blood (greater than 150 µL) was collected, during which the blood rapidly and regularly rose in the sampling device or in which blood fell in spontaneous and continuous drops into the tube. A problematic collection referred to first-time access failure, slow or irregular blood flow, insufficient volume of blood collected (less than 150 µL), or bleeding from the ear (in the case of facial vein phlebotomy).

Blood specimen analysis. Specimens were analyzed at the Laboratoire Central de Biologie Médicale de l'Ecole Nationale Vétérinaire de Toulouse within 2 h of sampling without refrigeration. Two blood smears were performed, stained using a May Grünwald-Giemsa automatic stainer (Aerospray Hematology Slide Stainer Cytocentrifuge 7150, Wescor, Logan, UT) and stored in an airtight box until microscopic evaluation.

Scoring platelet aggregation. Semiquantitative scoring of platelet aggregation of one smear was performed by the same trained histologist (NBA). The score was obtained by counting aggregates of more than 10 platelets over all the edges of the

smear (head, borders, and tail) at 200× magnification (Nikon 50i Eclipse microscope, Kobe, Japan). Scores were as follows: 4, more than 30 aggregates; 3, 21 to 30 aggregates; 2, 11 to 20 aggregates; 1, 6 to 10 aggregates; 0.5, 1 to 5 aggregates; 0, no aggregates. According to the threshold of 2 previously reported in feline hematology studies,[22,39] these scores were then grouped into 3 classes: nonaggregated, mildly aggregated, and highly aggregated, corresponding to scores of 0, 0.5 to 2, and greater than 2, respectively.

Hematology. Before analysis, specimens were placed on a rotary agitator for 20 min (Speci-Mix, model CT06478, Thermo Fisher Scientific, Langenselbold, Germany), and careful pipetting was performed to exclude any macroscopically visible clot. All tubes were analyzed without dilution or refrigeration on an ProCyte Dx analyzer (model 00-25-18, IDEXX, Westbrook, ME) using the corresponding mouse settings. The full CBC analysis was performed but only the 4 following variables were further investigated: platelet counts according to optical and impedance measurements, WBC count, and impedance RBC count. Quality controls of the analyzer were performed using the corresponding manufacturer's control solutions (e-check, XS, L1, and L2 IDEXX ProCyte Dx Quality Control).

Statistical analysis. Because the mice were randomly selected at each of the 3 experimental steps, which were separated by 6-wk washout periods, the results were considered to be independent. Statistical analysis was thus performed on pooled results obtained in the 3 studies, classified on the basis of collection site, sampling method, and platelet antiaggregation additive. Descriptive statistics (median and range) were obtained for aggregation scores, and platelet (optical and impedance), WBC, and RBC (impedance) counts. Possible effects of platelet aggregation inhibitors, sampling procedure, collection site, and the sampling process were tested according to multivariate ANOVA. Differences between subgroups were tested by applying the Mann–Whitney test. Effects were deemed statistically significant with $P < 0.05$. Calculations were made by using an Excel spreadsheet (Microsoft, Redmond, WA), Analyze-It (Analyze-It, Leeds, United Kingdom), Systat 13 (SyStat, Chicago, IL).

Results

Among the 80 mice originally enrolled, some specimens ultimately could not be used for various reasons: insufficient volume of blood available, macroscopically visible clots, and euthanasia of mice ($n = 2$ mice with head tilt or spinning, $n = 2$ mice with wounds and dermatitis). The number of specimens used in each study is listed in Table 1.

The subjective quality of the sampling process was estimated to be unremarkable in 80.3% of the cases, significantly ($P < 0.001$) higher than the 19.7% problematic samplings. Quality did not differ according to sex ($P = 0.322$), nor did the percentage of unremarkable samplings differ between the facial and jugular vein methods ($P = 0.433$).

Regarding platelet aggregation, 49% of specimens were nonaggregated, 35% were mildly aggregated, and 16% were highly aggregated. The aggregation score was significantly ($P < 0.001$, Mann–Whitney test) lower for specimens resulting from unremarkable sampling than from problematic sampling, with respective mean scores of 0.6 and 1.7. In addition, the aggregation score was lower for male mice than for female mice, with respective means of 0.60 and 1.18 ($P = 0.002$), and was higher in specimens from the facial vein than the jugular vein, with respective means of 1.22 and 0.42 ($P < 0.001$).

Taking into account all specimens of the 3 studies, platelet aggregation had no effect on RBC or WBC counts ($P = 0.195$ and 0.475, respectively) and was associated with significantly lower optical and impedance platelet counts ($P < 0.001$ and = 0.001, respectively; ANOVA) in highly aggregated specimens as compared with nonaggregated or mildly aggregated specimens (Figure 1).

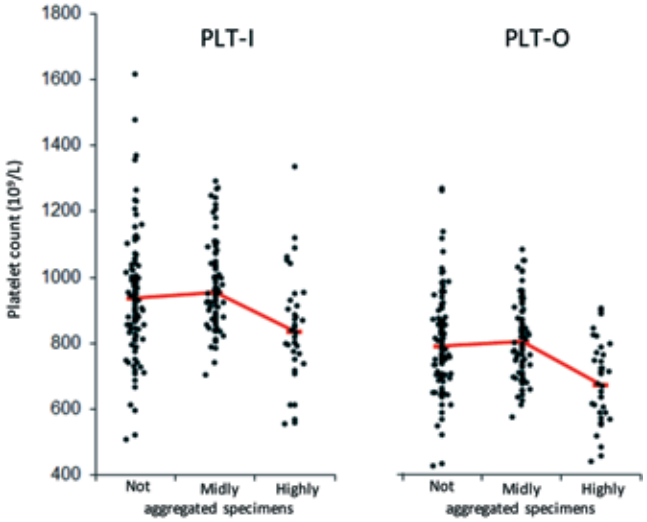

Figure 1. Comparison of platelet counts by impedance (PLT-I) and optical (PLT-O) methods in nonaggregated, mildly and highly aggregated EDTA-treated mouse blood specimens. The red line connects the median platelet counts of the 3 subgroups.

The aggregation scores and blood cell counts for all the associations tested between collection site, collection method, platelet aggregation inhibitor, and mouse sex are compared in Table 1. Neither the sampling method nor antiaggregant additive had any significant effect on platelet aggregation score or blood cell counts. In most cases, the effects of platelet aggregation were the same as for the overall results: most of the differences were not statistically significant, the main effect of the covariables was higher platelet counts in male mice than in female. In specimens sampled from the jugular vein and supplemented with iloprost, the platelet aggregation score was not significantly higher than for NaCl specimens and platelet counts were significantly lower ($P = 0.11$ and <0.001, respectively), whereas RBC and WBC counts were not changed.

Discussion

As stated in the introduction, possible preanalytical effects of the quality of nonterminal blood sampling in mice have rarely been investigated. The criteria chosen for assessing the quality of specimens collected in this study were no or only slight alteration of hematology variables due to sampling, with special emphasis on platelet aggregation and consequences on blood cell counts. These criteria may be considered as exceeding strict requirements if only platelet counts are altered and platelet counts are not a variable of interest in a study. However, stricter requirements concerning the quality of the specimen help to ensure accurate results for all variables.

The experimental protocol was based on a trial-and-error methodology in which platelet antiaggregant additives were tested first but did not demonstrate satisfactory efficacy. Second, the possible effects of different blood retrieval methods were compared and revealed fewer platelet aggregates in specimens collected from the jugular vein than for the facial vein. A last attempt was made to investigate the efficacy of a prostacyclin analog in blood specimens collected from the jugular vein, but it was as ineffective as it had been in specimens collected from

Table 1. Aggregation scores and blood cell counts (median [minimum–maximum]) of EDTA-treated blood specimens from 80 C57BL6/j mice

Collection site	Antiaggregant additive	Collection method	Sex	*n*	Aggregation score	PLT-O ($\times 10^9$/L)	PLT-I ($\times 10^9$/L)	WBC ($\times 10^9$/L)	RBC ($\times 10^{12}$/L)
Facial vein									
	Aspirin	Minivette	F+M	18	—	677 (459–793)	829 (558–981)	5.7 (3.4–9.2)	9.8 (9.5–10.3)
			F	8	4 (0.5–4)	—	—	—	—
			M	10	0.5 (0–4)	—	—	—	—
	Cangrelor	Minivette	F+M	18	0 (0–4)	—	—	—	9.8 (8.6–10.4)
			F	8	—	644 (426–696)	763 (521–879)	5.7 (3.1–7.8)	—
			M	10	—	738 (678–875)	857 (816–1037)	7.4 (5.7–11.7)	—
	Iloprost	Minivette	F+M	18	0 (0–4)	—	—	5.2 (3.5–9.7)	9.7 (8.8–10.3)
			F	9	—	577 (434–650)	711 (510–775)	—	—
			M	9	—	706 (612–817)	806 (667–962)	—	—
	Control (NaCl)	Minivette	F+M	39	0.5 (0–4)	—	—	6.1 (3.1–12.8)	—
			F	19	—	703 (484–859)	886 (571–1042)	—	9.7 (8.8–10.1)
			M	20	—	807 (220–1028)	953 (262–1338)	—	9.8 (4.2–10.7)
		Drop	F+M	20	1 (0–4)	—	—	—	9.6 (9.0–10.3)
			F	10	—	776 (670–847)	947 (796–1009)	4.5 (3.1–8.1)	—
			M	10	—	901 (441–1033)	1046 (557–1248)	8.8 (5.7–13.5)	—
		Microvette	F+M	20	1 (0–4)	—	—	—	9.6 (8.5–10.5)
			F	10	—	832 (559–800)	990 (613–1029)	5.8 (3.7–8.8)	—
			M	10	—	923 (829–1054)	1135 (985–1291)	8.3 (5.1–10.1)	—
Jugular vein									
	Iloprost	Needle and syringe	F+M	38	0 (0–4)	—	—	5.2 (0.6–13.2)	—
			F	19	—	750 (522–941)	858 (597–1036)	—	8.9 (6.9–9.7)
			M	19	—	825 (744–1079)	932 (867–1208)	—	9.1 (6.0–9.7)
	Control (NaCl)	Needle and syringe	F+M	57	0 (0–4)	—	—	5.5 (1.4–15.2)	—
			F	28	—	807 (548–961)	1097 (879–1618)	—	9.0 (7.1–9.8)
			M	29	—	932 (700–1271)	953 (613–1198)	—	9.1 (7.0–9.7)

F, female; M, male; PLT-I, platelet count by impedance method; PLT-O, platelet count by optical method.

the facial vein. Although these trial-and-error tests did not allow us to select optimal preanalytical conditions for hematology analyses in mice, they did demonstrate that some procedures should not be used. They also showed that quality scoring of the sampling process can be a useful predictor— probably the key point—of the quality of the blood specimen.

In this study, the quality of the sampling process was considered to be unremarkable in 80% of the cases, with no significant difference between the facial vein and jugular vein collection sites. However, blood flow from the facial vein was unpredictable and differed markedly from one mouse to another. When the blood flow was abundant, the capillarity devices (Minivette and Microvette) missed some drops, whereas an insufficient flow rate led to premature closure of the puncture site and insufficient blood collected. Other adverse events associated with sampling the facial vein included intermittent bleeding from the ear or nose, and 2 mice developed vestibular syndrome because of ear hematoma within few minutes after sampling. Our results may underestimate adverse events because the loss of one or 2 drops of blood was not considered to be problematic and, because vestibular syndrome appeared after sampling, it was not considered equivalent to 'problematic sampling'. These negative effects are in contrast to previous studies that reported no adverse effects of facial vein phlebotomy in mice[15] or those that reported fewer adverse effects when using the facial vein than the jugular vein.[54] However, an acute increase in plasma corticosterone concentrations after sampling blood from the facial vein was reported in a study focused on the influence of blood sampling technique on mouse welfare.[36] Sampling from the jugular vein has never been associated with macroscopic hematoma or as having a behavioral effect on mice. The rough estimation of the sampling process used in this study (that is, problematic compared with unremarkable) should now be improved by using more fact-based scoring, given that the quality of the sampling was clearly predictive of the quality of the resulting specimen—the platelet score of aggregation was more than 2.5 times higher in problematic as compared with unremarkable samplings. This difference underlines the need to pay careful attention to accurately recording the sampling conditions to avoid possible analytical errors.

According to the platelet aggregation score, the quality of the specimens from the jugular vein was better than that of the facial vein, as the score for jugular samples was almost a third that of the facial vein. Platelet counts, measured by impedance and optical methods, were lower in highly aggregated specimens, whereas they were unaltered in mildly aggregated specimens. Platelet clumps are considered to invalidate platelet counts,[14] leading some authors to not report platelet

counts to avoid artefactually low platelet counts.[37,40] Pseudothrombocytopenia secondary to platelet clumping during preanalytical and analytical processes is a well-known issue in human clinical pathology, leading to diagnostic errors and unnecessary medical treatment.[32] Such spurious results have also been described in cats and minipigs, both of which are particularly prone to platelet aggregation.[13,22,39] As in the current study, the sampling process was the same for both sexes. The more numerous platelet aggregates and lower platelet counts observed in females could therefore not have only been due to blood sampling difficulties related to the lower body weight of females, as previously reported.[49] A sex-associated effect on platelet counts was previously reported in mice, with higher platelet counts in male than in female C57Bl/6J mice.[2] In humans, a recent publication reports higher platelet reactivity in women,[44] partly due to estrogen-induced increased platelet reactivity.[41] In our current study, neither the number of aggregates nor the sampling site affected RBC or WBC counts, in disagreement with previous publications on mice,[38] cats,[39] and humans[48,57] that reported spurious leukocytosis due to EDTA-induced platelet clumping. This discrepancy could easily be due to the different technologies used to count the leukocytes. Impedance cell counters are known to overestimate leukocyte counts because platelet clumps mimic WBC.[58]

Inhibitors of platelet aggregation that are reported to be effective under in vitro conditions in humans,[3,4,17,20,23,35,59] rabbits,[56] cats[46,55] and mice[7,10,11,29] had no effect on the mouse blood specimens in our current study, regardless of the collection site or the sampling method. We expected the greatest effects from iloprost, a stable prostacyclin analog that binds to specific surface receptors on platelets, thus reducing their capacity to aggregate. However, iloprost added to mouse EDTA-treated blood specimens did not reduce platelet aggregation. This finding could be disappointing for studies that require accurate platelet counts. However, the proportion of nonaggregated or mildly aggregated specimens that we obtained in this study suggests that experienced and careful samplers are probably more important than the use of antithrombotic agents and corroborates the basic principle underlying blood sampling for scientific purposes: "the more routinely blood sampling is performed, the better the quality of the blood taken and the less stressful the sampling procedure will be to the animal."[51]

In summary, sampling blood from the jugular vein of mice under general anesthesia is the best compromise between animal welfare and high-quality nonterminal blood specimens; we particularly recommend jugular sampling if platelet counts are to be measured. Adding currently used antiaggregants does not effectively reduce the platelet aggregation that leads to spuriously low platelet counts and also does not affect RBC (impedance) and WBC counts with the ProCyte Dx analyzer and likely other equipment that uses the same technology. Unless another inhibitor of platelet aggregation is shown to be effective, particular attention should be given to optimizing sampling conditions and to scoring platelet aggregates when necessary.

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