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Clinical Biochemistry Parameters in C57BL/6J Mice after Blood Collection from the Submandibular Vein and Retroorbital Plexus

Itziar Fernández,[1] Arantza Peña,[1] Nahia Del Teso,[1] Virginia Pérez,[1] and Juan Rodríguez-Cuesta[1,*]

Collection of blood from the submandibular vein allows simple and rapid processing of many animals without anesthesia and facilitates rapid recovery with no signs of pain and discomfort in the mice. Here we compared the submandibular vein and retroorbital plexus blood collection methods, to determine the potential effect of the sampling technique on several clinical biochemistry parameters in C57BL/6J mice. We found statistically significant differences for 8 of the 9 biochemical parameters studied between the 2 blood sampling techniques. Compared with samples collected from the retroorbital plexus, blood obtained from the submadibular vein had higher levels of AST, ALT, protein, albumin, triglycerides, total cholesterol, and creatinine. Glucose values of retroorbital blood were higher than those from the submandibular vein. Urea levels were similar for both sampling techniques. Our results demonstrate that the technique used to obtain blood samples affects parameters commonly used to assess animal health. We recommend caution when comparing results of biochemical analysis of blood obtained from the submandibular vein in mice with reference values obtained by other blood sampling techniques.

Blood for biochemical analysis can be obtained in mice by various techniques, including bleeding from the retroorbital plexus (also described as the retrobulbar venous plexus and periorbital sinus), by the tailclip technique, by cardiocentesis, and by saphenous venipuncture.[7,9,10] Each method can affect the outcome of serum biochemistry analysis, due to differences in handling, restraining, anesthesia, invasiveness, and animal discomfort.[1,13,19,20] The retroorbital blood-collection method is widely used in mice.[8,9,13] Although this method consistently yields a reasonable blood volume when the investigator is experienced in the procedure, retroorbital blood collection is controversial because it may cause pain, distress, or even blindness when performed incorrectly.[21,22] A joint working group on refinement does not recommend retroorbital sampling because of the risk of tissue damage[12] and states that this method is acceptable only as a terminal procedure while the animal is anesthetized.[17]

Recently, new blood sampling methods considered more humane and less aggressive than the retroorbital technique, such submandibular venipuncture, have been developed in mice.[8] Although several reports[16,19] address the retroorbital sampling method, detailed information regarding submandibular venipuncture is scarce in the published literature. The goal of our study was to compare the retroorbital technique of blood collection with submandibular venipuncture to determine the effect of this new bleeding method on several clinical biochemistry parameters in C57BL/6J mice.

Materials and Methods

Animals. All the animal procedures were carried out at our AAALAC-accredited animal facility (CIC bioGUNE, Biscay, Spain) and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*[11] and European policies.[5] The animal colony was screened quarterly and tested negative for: minute virus of mice, mouse parvovirus, mouse hepatitis virus, pneumonia virus of mice, reovirus 3, Sendai virus, mouse rotavirus, mouse norovirus, mouse thymic virus, mouse cytomegalovirus, Hantaan virus, lymphocytic choriomeningitis virus, Theiler virus, mouse adenovirus, K virus, ectromelia virus, polyoma virus, lactic dehydrogenase virus, *Clostridium piliforme*, *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Pasteurella* spp., *Salmonella* spp., *Streptobacillus moniliformis*, β-hemolytic *Streptococcus*, *Streptococcus pneumoniae*, *Helicobacter* spp., cilia-associated respiratory bacillus, ectoparasites, *Encephalitozoon cuniculi*, and pathogenic protozoa and helminthes. C57BL/6J male mice (age, 6 wk; n = 20) were obtained from Charles River Laboratories (L'Arbresle, France) and housed in groups of 5 in polycarbonate cages containing woodchip bedding (Lignocel, J Rettenmaier and Söhne, Rosenberg, Germany) in a room with controlled temperature (20 to 24 °C) and relative humidity (50% to 65%) and a 12:12-h dark:light cycle (lights on 0800 to 2000). Mice were fed rodent maintenance diet (2014, Harlan Teklad, Barcelona, Spain) and provided with water ad libitum. The protocol was approved by the Bioethical and Animal Welfare Committee of CIC bioGUNE (code P-CBG-CBBA-0307).

Experimental design. Mice were assigned randomly to 2 groups (SM and RO) of 10 each and were acclimated for 2 wk before the first blood extraction. When mice were 8 and 16 wk old, blood samples were obtained by the retroorbital method from each animal in the RO group first and then by submandibular venipuncture from each animal of the SM group. When mice were 22 wk old, retroorbital samples were obtained from the mice that previously had undergone submandibular venipuncture and vice versa. This approach was used to control for possible intrinsic variations in the parameters under study among the mice.

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[1]Animal Unit, Biosciences Cooperative Research Centre (CIC bioGUNE), Biscay, Spain.
[*]Corresponding author. Email: jrodriguez@cicbiogune.com

Table 1. Serum biochemistry parameters in mice by age and collection method

Parameter	Collection method	8 wk				16 wk				22 wk			
		n	Mean	SD	95% Confidence interval	*n*	Mean	SD	95% Confidence interval	*n*	Mean	SD	95% Confidence interval
AST (IU/L)													
	SM	10	76.19	17.71	65.21–87.17	9[a]	56.07	14.14	47.31–64.84	9[a]	57.73[b]	12.55	49.95–65.51
	RO	10	67.13	25.81	51.13–83.13	10	63.49	19.33	51.51–75.47	10	42.27	7.45	37.65–46.89
ALT (IU/L)													
	SM	9[a]	27.00[b]	6.16	23.18–30.82	9[a]	28.79	9.00	23.21–34.37	9[a]	21.78[b,c]	6.36	17.84–25.72
	RO	10	23.00	4.92	19.95–26.05	10	27.38	8.34	22.21–32.55	10	14.67	2.65	13.03–16.31
Glucose (mg/dL)													
	SM	10	260.61	48.02	230.85–290.37	10	225.66[b,c]	26.49	209.24–242.08	10	237.08[b]	54.65	203.21–270.95
	RO	10	277.98	42.58	251.59–304.37	10	276.90	58.91	240.39–313.41	10	290. 14	45.96	261.66–318.62
Protein (g/dL)													
	SM	10	5.326	0.514	5.007–5.644	10	5.865[b]	0.352	5.647–6.083	10	6.255[b]	0.353	6.036–6.473
	RO	10	5.077	0.207	4.949–5.205	10	5.267	0.306	5.078–5.457	10	5.402	0.167	5.299–5.506
Albumin (g/dL)													
	SM	10	3.769	0.379	3.534–4.004	10	4.181[b]	0.353	3.962–4.400	10	4.419[b]	0.327	4.216–4.621
	RO	10	3.549	0.145	3.459–3.639	10	3.642	0.241	3.493–3.792	10	4.012	0.154	3.917–4.108
Triglycerides (mg/dL)													
	SM	10	92.25	12.22	84.67–99.83	9[a]	92.74[b]	9.56	86.82–98.67	10	96.28[b]	15.87	86.45–106.11
	RO	10	82.08	13.75	73.56–90.60	10	74.34	15.87	64.51–84.17	10	73.39	6.40	69.43–77.35
Total cholesterol (mg/dL)													
	SM	10	111.53	13.17	103.37–119.69	10	124.57[b]	12.88	116.59–132.55	10	149.37[b]	20.76	136.50–162.24
	RO	10	103.92	7.79	99.09–108.75	10	108.05	9.93	101.90–114.20	10	125.79	12.47	118.06–133.52
Creatinine (mg/dL)													
	SM	10	0.53[b,d]	0.03	0.51–0.55	10	0.53[b]	0.04	0.50–0.56	10	0.52[b]	0.05	0.49–0.55
	RO	10	0.38	0.03	0.36–0.40	10	0.41	0.04	0.39–0.44	10	0.46	0.08	0.41–0.51
Urea (mg/dL)													
	SM	10	44.47	4.96	41.39–47.54	10	48.41	6.43	44.42–52.39	9[a]	52.04	5.35	48.72–55.35
	RO	10	40.14	3.34	38.07–42.21	9[a]	50.61	8.47	45.36–55.85	10	52.86	8.83	47.39–58.33

SM, submandibular venipuncture; RO, retroorbital collection method.
[a]1 outlier removed.
[b]Significantly different ($P < 0.05$) from RO value.
[c]Statistical comparison based on Welch test.
[d]Statistical comparison based on Mann–Whitney rank sum test.

Blood sampling method and sample handling. Blood samples were always collected during the same time interval in the afternoon (1500 to 1530) after a fasting period of 5 h. Submandibular blood samples were obtained by incising the right submandibular vein of unanesthetized mice with a sterile 4-mm lancet (MediPoint, Mineola, NY). Retroorbital blood samples were collected from the right retroorbital plexus of anesthetized mice. Anesthesia was induced by placing each mouse in an inhalation chamber with 4% isoflurane (IsoFlo, Abbott Laboratories, Berkshire, UK) regulated with a calibrated vaporizer. Blood samples were deposited in serum separator gel tubes (Microtainer, Becton–Dickinson, Franklin Park, NJ) and centrifuged (9,300 × *g*, 30 min, 4 °C) for serum separation. Serum hemolysis was evaluated by direct observation and recorded as follows: 0, no hemolysis; 1, slight; 2, moderate; and 3, severe hemolysis. The volume of each blood sample was approximately 300 µL, and at no time did this volume exceed that recommended for mice in regard to body weight and recovery time.[7] Mice were allowed to recover completely on a regulated thermal blanket after each bleeding session and were observed daily for signs of pain or discomfort.

Clinical chemistry parameters. Serum activity AST and ALT and concentrations of triglycerides, glucose, total cholesterol, total protein, albumin, urea, and creatinine were determined by using an automated analyzer (Selectra Junior Spinlab 100, Vital Scientific, Dieren, Netherlands; Spinreact, Girona, Spain) according to the manufacturers' instructions. Standard controls were run before each determination, and the values obtained for the different biochemical parameters were always within the expected ranges. The intraassay variability of biochemical assays was relative to 12 repeated determinations of the control serum in the same analytical session, whereas interassay variability for each parameter was calculated on the mean values of control sera measured during 6 analytical sessions.

Statistical analysis. To identify the optimal number of mice, a power analysis for determining sample size (SPSS 10.0, SPSS, Chicago, IL) was conducted with an alpha value of 0.05 and a power of 90%. Our laboratory's historical data were used to establish an expected difference in means and an expected SD for each biochemical parameter to perform this calculation.

For each biochemical parameter and time point, the group mean, SD, and 95% confidence interval were calculated for both

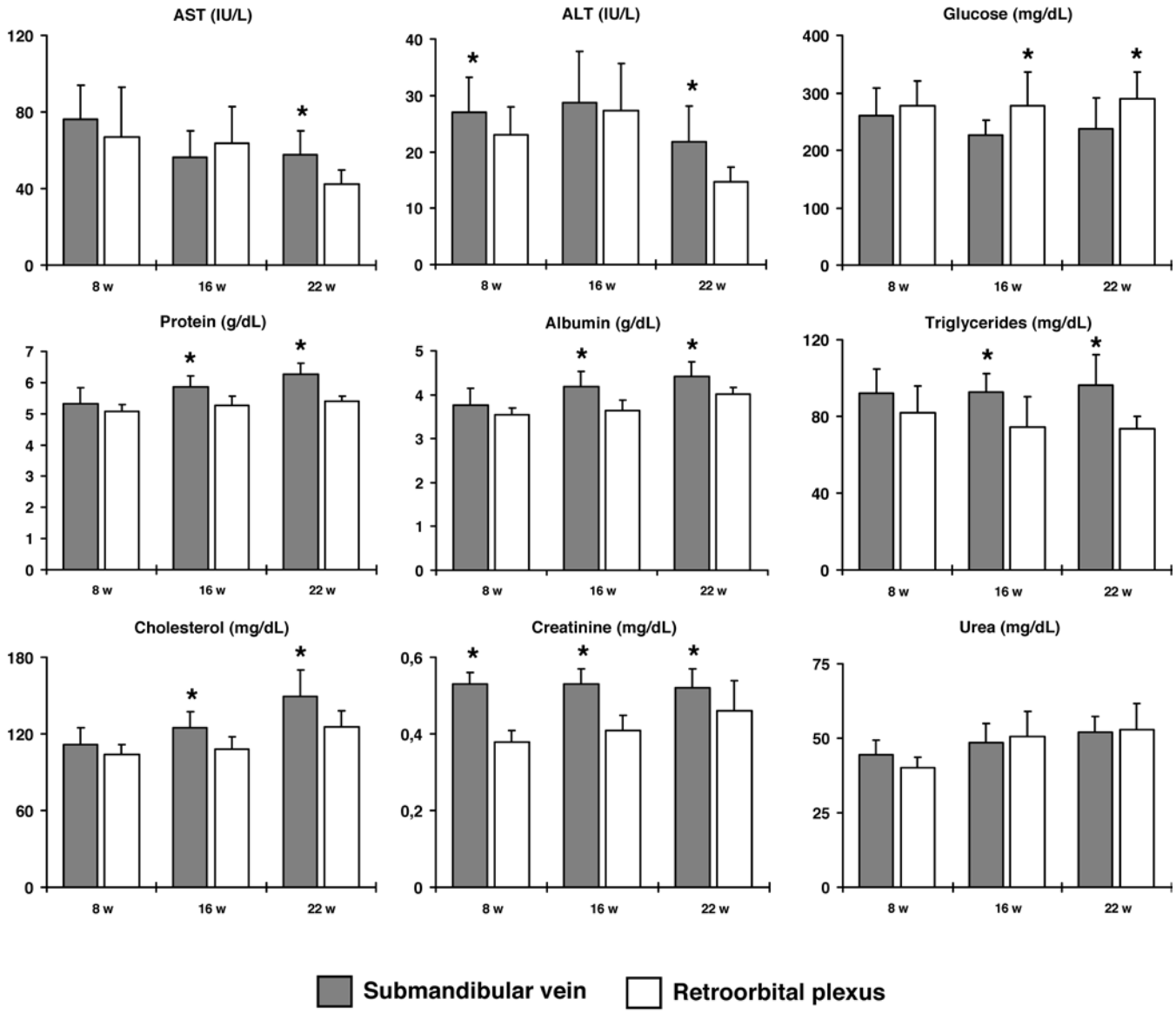

Figure 1. Comparison of clinical biochemistry parameters in mice by age and collection method. Values (mean ± SD) for 9 parameters measured in the serum of C57BL/6J mice at 8, 16, and 22 wk of age by using submandibular (dark bars) and retroorbital (white bars) collection methods. Statistically significant differences (*, $P < 0.05$) observed among means are indicated.

blood sampling methods. Statistical analyses were performed (SPSS 10.0, SPSS) and included tests of normality (Kolmogorov–Smirnov) and equal variance (Levene median). The Grubb test (GraphPad Software, La Jolla, CA) was run for outlier detection. Outliers were removed, and group means, SD, and CI were recalculated.

To compare data between groups (SPSS 10.0, SPSS), the Student *t* test (parametric) was used when conditions of normality and equal variance were met. The Student *t* test was used with Welch correction when unequal variances were detected. When the normality test failed, a 2-tailed and exact Mann–Whitney rank sum test (nonparametric) was used. Differences were considered statistically significant at a *P* value of less than 0.05.

Results

Hemolysis level. A mean hemolysis value was calculated for the SM and RO groups at each time point. Hemolysis did not differ significantly between SM and RO groups at 8 wk (0.6 and 0.2, respectively), 16 wk (0.6 and 0.3, respectively), or 22 wk (0.4 and 0.1, respectively).

Clinical chemistry parameters. Intra- and interassay variability of analytical assays (expressed as coefficients of variation) were always less than 4% for all biochemical parameters (data not shown). Age-related statistical data for biochemical parameters derived from both blood sampling techniques are presented in Table 1. At 8 wk, blood obtained by submandibular venipuncture had significantly ($P < 0.05$) higher mean levels of ALT and creatinine compared with samples collected by retroorbital technique (Figure 1). Identical analyses performed at 16 wk revealed that blood collected from the SM site had significantly ($P < 0.05$) higher levels of protein, albumin, triglycerides, total cholesterol, and creatinine and significantly lower ($P < 0.05$) glucose than did blood collected from the RO site.

At 22 wk of age (when mice were sampled from the alternate site), blood obtained from the SM site had higher ($P < 0.05$) levels of AST, ALT, protein, albumin, triglycerides, total cholesterol,

and creatinine and lower glucose than did blood from the RO site. However, no difference between methods was found at any time point for urea.

Discussion

Many sources of variation can affect the results of clinical biochemistry assays.[2,4,6,15,16,18,23] These alterations can result from events that occur prior to sample collection, such as the fasting condition or environmental stress. The blood sampling process itself is a source of variation, including the collection site, anesthesia used, level of hemolysis, and the skill of the technician performing the procedure. Finally, factors during the period that follows blood extraction may influence biochemical parameters and include tube selection, processing delay, and the analytical procedure itself. Additional factors, such as gender, age, and strain, also are known sources of variation in biochemical values. In light of the ability of the sampling method to alter assay results, here we focus on the effect of the new submandibular blood collection method on 9 biochemistry parameters in C57BL/6J mice.[1,13,19,20] The 3 time points considered in this study (8, 16, and 22 wk of age) were chosen because most of published data about mice biochemical physiology refer to these ages.[3,14]

We found statistically significant differences between the 2 blood sampling techniques for 8 of the 9 biochemical parameters studied. Seven biochemical parameters (AST, ALT, triglycerides, total cholesterol, protein, albumin, and creatinine) showed small but statistically significant higher mean values in serum obtained by submandibular venipuncture compared with retroorbital blood collection. In contrast, serum collected from the retroorbital demonstrated significantly higher mean glucose values than that obtained from the submandibular vein. Urea concentration was unaffected by sampling technique at the 3 time points studied.

The blood sampling in this work was performed by the same skilled technician for all samples and time points, and all the actions performed before and after blood collection were standardized, so that variability due to these reasons could be excluded from our results. Therefore, we assume that the differences in the values assessed reflect factors directly associated with the blood sampling method, including handling stress, anesthesia, hemolysis, and tissue damage. That changing the bleeding technique between groups yielded similar results strongly suggests that the mean differences observed are associated directly with the blood collection technique used, rather than due to intrinsic variation of biochemical parameters among animals. Stress associated with the handling of mice during the bleeding process increases the glucose serum concentrations proportionally to the handling time.[2] This situation may explain the substantial increase in glucose concentration in the RO group during our study: retroorbital blood sampling involves extensive handling, primarily because of anesthesia. In addition, the lower protein and albumin concentrations obtained after retroorbital blood collection may reflect effects of the isoflurane anesthesia used for this bleeding method. In line with this argument, total protein and albumin levels were lower in rats anesthetized with isoflurane in comparison with animals that did not receive anesthesia.[6]

In the present study, the level of hemolysis in all serum samples was scored by direct observation. Increased hemolysis has been proposed to contribute to differences in some biochemical parameters, including AST and ALT.[16] The tissue damage that may occur during the sampling process is another factor that may contribute to differences between values obtained by different collection techniques. Additional studies should address the effects that these and other variables, including as mouse gender and strain, exert on samples obtained by submandibular venipuncture.

After 2 y of experience with the technique in our facility, we consider submandibular venipuncture much easier to perform than retroorbital blood collection. The submandibular bleeding method allows relatively rapid processing of many animals without anesthesia, thereby eliminating the potential effects of an anesthetic on animal physiology and the potential risk of ocular trauma. In addition, submandibular venipuncture accommodates the use of the same animal at multiple time points, facilitates rapid recovery, and is not associated with signs of pain and discomfort in mice after blood collection. In light of these observations, we now use submandibular venipuncture when repeated blood samples from mice are needed. We recommend the retroorbital method only as a terminal procedure.

The differences observed between the 2 sampling techniques we assessed suggest the necessity for individual laboratories to establish their own reference ranges for clinical biochemical parameters in mice, according to their routine analytical procedures. We recommend caution when biochemical parameter results obtained by submandibular venipuncture in mice must be compared with reference values obtained through other blood sampling techniques. Submandibular venipuncture clearly contributes to refinement of experimental technique, in accordance with ethical and legal regulations regarding the use of animals in biomedical research.[5]

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