SHOCK, Vol. 63, No. 6, pp. 944–946, 2025

OPEN

Brief Report

CREATINE KINASE IS ELEVATED BY THE SUBMANDIBULAR VEIN BLEED TECHNIQUE, OBSCURING THE MEASUREMENT OF MUSCLE DAMAGE IN SEPSIS

Taniah Ali,[1] Daniela Rodarte,[1] Luiz F. Garcia,[1] Sydney Ligon,[1] Chander Khatri,[1] and Wendy E. Walker[2]

[1]Department of Molecular and Translational Medicine, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center (TTUHSC) El Paso, El Paso, Texas; [2]Department of Biomedical Sciences, Mercer University School of Medicine, Columbus, Georgia

Received 24 Jan 2025; first review completed 19 Feb 2025; accepted in final form 4 Mar 2025

KEYWORDS—Creatine kinase; organ damage; submandibular bleed; retro-orbital bleed; sepsis; cecal ligation and puncture; animal welfare

Organ dysfunction is a defining feature of sepsis (per the sepsis-3 guidelines (1)), and it is therefore key to measure organ dysfunction in animal models of sepsis. For nonterminal studies, organ damage can be assessed by measuring serum enzymes, including creatine kinase (CK; indicating muscle damage), lactate dehydrogenase (LD, indicating tissue damage), and blood urea nitrogen levels (BUN, indicating impaired kidney function), as shown by our own lab and others (2–10). When designing an animal research study, it is always important to consider the three Rs: replacement, reduction, and refinement. Regarding the latter, prior reports suggest that it may be preferable to obtain blood from the submandibular vein of mice *versus* the retro-orbital plexus for improved animal welfare, depending on the experimental design and the experience level of the investigator (11–13). During the course of our studies, we switched from the retro-orbital bleed technique to the submandibular vein bleed technique, as an effort to refine our humane use of animals. However, we encountered an unexpected effect on the measurement of serum CK, an indicator of muscle damage, which we describe in this brief report.

This study aimed to investigate the effects of sleep interruption on sepsis pathogenesis. Animal experiments were approved by the IACUC of TTUHSC El Paso and Mercer University. Male and female C57BL/6J mice (5–15 weeks old) were subjected to a subject to a sleep interruption procedure in which they were housed on an intermittent orbital rotator, as described in a previous report (14). The equipment was set to oscillate at 100 RPM for 30 s, and then switch off for 90 s. This cycle was repeated every 2 min for 48 h starting at "lights on" in the 12-h light/12-h dark vivarium cycle. A second group of mice was subject to a control procedure in which they were housed on the same orbital shaker during the nighttime (when mice are normally awake) but returned to their stationary housing mode during the daytime (when mice sleep), for 48 h in the same manner. Another control group of mice was allowed to sleep normally (they were never placed on the orbital shaker). All three groups received hydrogels for the 48-h period. The animals tolerated these procedures well with no overt signs of distress.

The animals subsequently underwent cecal ligation and puncture (CLP) to induce sepsis (1 cm ligation, 2 punctures) and received lactated ringer's solution and imipenem/cilastatin antibiotics, per our prior reports (3,4,15–17). Blood was collected at 0 h (pre-operatively), 6 h, 12 h, and 24 h relative to the procedure. Blood samples were collected from the retro-orbital plexus or submandibular vein. For both techniques, animals were anesthetized with a precision isoflurane vaporizer. For the retro-orbital bleed technique, proparacaine was applied to numb the eye, and then blood was collected by gently easing a capillary tube between the eye and the retro-orbital plexus and rotating it. Gentle pressure was applied to stop the bleeding, and ophthalmic ointment was applied to soothe the eye. This procedure is described in detail in our prior report (18). To reduce the stress to the animals, we applied a soothing petroleum ophthalmic ointment after each bleed, and alternated between the eyes for serial bleeds. For the submandibular vein bleed technique, the puncture site was located between the mandible and ear, slightly superior to the natural fur whorl on the mouse face. The vein was punctured with a lancet, then blood was collected with a capillary tube. Pressure was applied to stop the bleeding. We limited the total amount of blood collected to <10% blood volume as the sum of all bleeds. Animals were weighed pre-operatively to calculate their blood volume based on weight (using the formula 7.8 mL/100 kg body weight (19)).

The blood was spun down in gold-top BD microtainers with clot activator and serum was collected from above the barrier gel. Total CK, LD, and BUN levels were measured using reagents from Pointe Scientific. Data were compared with a Mann-Whitney test (pair-wise comparisons), or a Kruskal-Wallis test with Dunn's multiple comparison test (3+ groups). $P < 0.05$ was considered significant.

Address reprint requests to: Wendy E. Walker, PhD, 1633 1st Ave, Columbus, GA 31901. E-mail: walker_w@mercer.edu

This study was supported by a grant from the NHLBI (R15HL159554, awarded to WEW).

T.A., D.R., L.F.G., and S.L. contributed to this manuscript equally.

The authors report no conflicts of interest.

DOI: 10.1097/SHK.0000000000002585

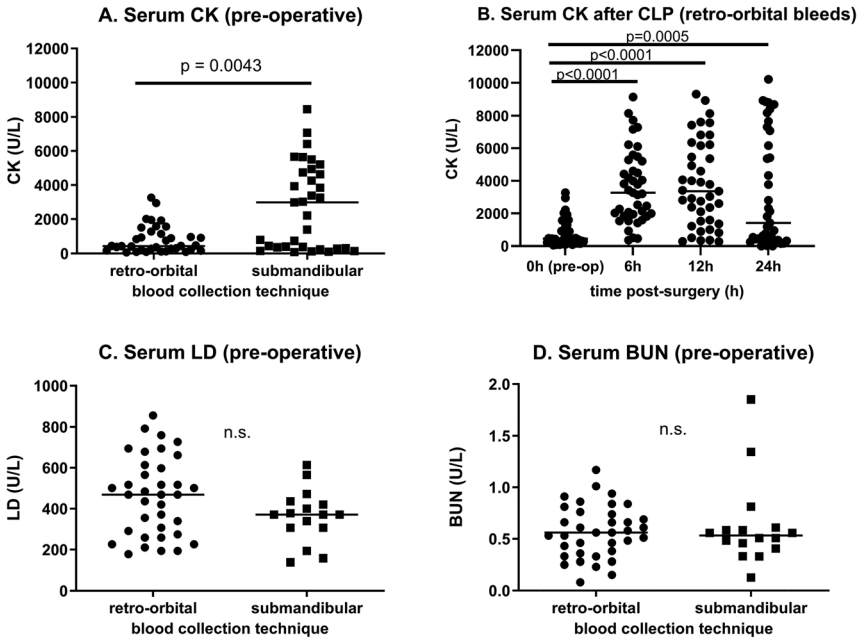

FIG. 1. **Submandibular bleeds elevate serum CK, obscuring muscle damage induced by sepsis.** Male and female C57BL/6 mice were subject to CLP to induce sepsis. Blood samples were obtained *via* the retro-orbital or submandibular route pre-operatively. Serum enzymes were measured including (A) CK (n = 36 for retro-orbital bleeds, n = 33 for submandibular bleeds), (C) LD (n = 36 for retro-orbital bleeds, n = 16 for submandibular bleeds) and (D) BUN (n = 36 for retro-orbital bleeds, n = 16 for submandibular bleeds). B, For the retro-orbital route, blood samples were obtained at 0 h (pre-operatively), and at 6 h, 12 h, and 24 h after CLP and we measured the elevation in CK induced by sepsis (n = 43 mice, with serial samples obtained from the same mice for each time point). BUN, blood urea nitrogen; CK, creatine kinase; LD, lactate dehydrogenase.

We observed no difference in CK levels between the "sleep interruption," "control procedure," and "normal sleep" groups included in our study (the comparison is not shown, for brevity). However, we obtained an unexpected finding that approximately half of the mice in the submandibular vein bleed technique showed an elevated serum CK level at baseline (e.g., for the pre-operative bleed) (Fig. 1A). The CK levels in the animals bled *via* the retro-orbital route ranged from 64 to 3,266 U/L, while CK levels in the mice bled *via* the submandibular vein route ranged from 87 to 8,450 U/L, with 15/33 exceeding the maximum value in the retro-orbital bleed group (Fig. 1A). Overall, the CK levels were significantly higher in the group bled from the submandibular vein in comparison to the animals bled from the retro-orbital plexus (Fig. 1A). Importantly, the baseline levels of CK in mice bled *via* the submandibular route were similar to those observed in septic mice bled *via* the retro-orbital route at 6, 12, and 24 h after surgery (Fig. 1B). In contrast to the results obtained with CK, the levels of baseline serum LD and BUN were similar in animals bled *via* the retro-orbital versus submandibular vein methods (Fig. 1, C–D).

Our data indicate that the lancet-based submandibular vein blood collection method elevated the serum CK readings in our study, in the blood samples obtained before sepsis was induced. We believe that this was most likely the result of local skeletal muscle damage when the cheek muscle was punctured by the lancet. Because the magnitude of this effect was similar to the elevation in serum CK that occurs 6–24 h post-CLP; it obscured the measurement of muscle damage due to sepsis, and prevented us from using these blood samples to compare our experimental groups. We present this as a cautionary tale for others interested to measure muscle damage in sepsis and related conditions. This effect could be averted by using a different blood collection technique, and possibly by refining the submandibular vein bleed technique. Notably, some investigators use a needle instead of a lancet to puncture the submandibular vein, and this could possibly minimize the elevation in CK but we have not tested this. We expect that this effect could also be avoided by measuring specific CK isoforms for certain applications (e.g., MM-muscle, MB-heart, BB-brain damage). Notably, the MB-heart isoform is used to measure myocardial injury in sepsis while the MM-skeletal muscle isoform defines rhabdomyolysis which also occurs during sepsis (5–7).

Regarding animal welfare, the mice tolerated both procedures well, with little signs of discomfort. No eye damage was observed with retro-orbital bleeds. A few mice bled from the ear after submandibular vein bleeds during our initial attempts at his procedure (an expected adverse effect for this bleed technique). We found that blood flow was easier to control with the retro-orbital method in comparison to the submandibular vein bleed method, minimizing blood loss. Notably, our team is highly experienced at the retro-orbital bleed technique; however, these findings were similar for our newer and more experienced lab members. To further reduce any harmful effects of repeated retro-orbital bleeds, one could reduce the number of blood samples collected per mouse, and this will also reduce the total blood loss. Furthermore, administration of fluids such as lactated ringer's solution postop can help to mitigate the effects of blood loss. Limitations of this study are that the data were generated from a single lab in only one mouse strain, and we have not performed histological analysis to confirm muscle damage from the lancet puncture, or to examine potential damage to the retro-orbital plexus from the retro-orbital bleeds.

In conclusion, retro-orbital blood collection may present some advantages over submandibular blood collection, depending on the research application and the experience level of the research team.

ACKNOWLEDGMENTS

We thank the veterinarians and animal care staff of Mercer University and TTUHSC El Paso for caring for these experimental mice.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA*. 2016;315(8):801–810.
2. Walker WE, Bozzi AT, Goldstein DR. IRF3 contributes to sepsis pathogenesis in the mouse cecal ligation and puncture model. *J Leukoc Biol*. 2012;92(6):1261–1268.
3. Heipertz EL, Harper J, Walker WE. STING and TRIF contribute to mouse sepsis, depending on severity of the disease model. *Shock*. 2017;47(5):621–631.
4. Goswami DG, Walker WE. Aged IRF3-KO mice are protected from sepsis. *J Inflamm Res*. 2021;14:5757–5767.
5. Guo L, Li P, Wang Y, et al. YIQIFUMAI injection ameliorated sepsis-induced cardiomyopathy by inhibition of ferroptosis via XCT/GPX4 Axis. *Shock*. 2024;61(4):638–645.
6. Teng Y, Li N, Wang Y, et al. NRF2 inhibits cardiomyocyte pyroptosis via regulating CTRP1 in sepsis-induced myocardial injury. *Shock*. 2022;57(4):590–599.
7. Delfrate G, Albino LB, Assreuy J, et al. Cecal slurry as an alternative model to CE-CAL ligation and puncture for the study of Sepsis-induced cardiovascular dysfunction. *Shock*. 2024;62(4):547–555.
8. Yu B, Weng L, Li J, et al. Inhibiting SIRT2 attenuates sepsis-induced acute kidney injury via FOXO1 acetylation-mediated autophagy activation. *Shock*. 2025;63(2):255–266.
9. Gong Y, Wei N, Shi P, et al. CIRCTMCO3 alleviates sepsis-induced acute kidney injury via regulating MIR-218-5P/ZEB2 axis. *Shock*. 2025;63(1):168–175.
10. Stolarski AE, Lai J-J, Kim J, et al. Genetic ablation of the c-type lectin receptor CLEC2D increases peritonitis Mortality, inflammation, and physiology without diminishing organ injury. *Shock*. 2024;62(3):437–446.
11. Regan RD, Fenyk-Melody JE, Tran SM, et al. Comparison of submental blood collection with the retroorbital and submandibular methods in mice (Mus musculus). *J Am Assoc Lab Anim Sci*. 2016;55(5):570–576.
12. Ahrens Kress AP, Zhang Y, Kaiser-Vry AR, et al. A comparison of blood collection techniques in mice and their effects on welfare. *J Am Assoc Lab Anim Sci*. 2022;61(3):287–295.
13. Hoggatt J, Hoggatt AF, Tate TA, et al. Bleeding the laboratory mouse: not all methods are equal. *Exp Hematol*. 2016;44(2):132–137.e1.
14. Sinton CM, Kovakkattu D, Friese RS. Validation of a novel method to interrupt sleep in the mouse. *J Neurosci Methods*. 2009;184(1):71–78.
15. Goswami DG, Rubio AJ, Mata J, et al. Large peritoneal macrophages and transitional premonocytes promote survival during abdominal sepsis. *Immunohorizons*. 2021;5(12):994–1007.
16. Goswami DG, Walker WE. Detection of blood cell surface biomarkers in septic mice. *Methods Mol Biol*. 2021;2321:191–205.
17. Garcia LF, Singh V, Mireles B, et al. Common variables that influence sepsis mortality in mice. *J Inflamm Res*. 2023;16:1121–1134.
18. Walker WE. Methods to study the innate immune response to sepsis. *Methods Mol Biol*. 2018;1717:189–206.
19. O'Connell KE, Mikkola AM, Stepanek AM, et al. Practical murine hematopathology: a comparative review and implications for research. *Comp Med*. 2015;65(2):96–113.