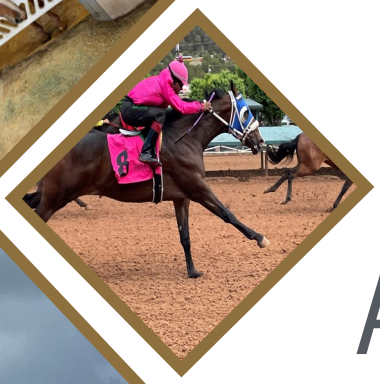

ANNUAL REPORT

Prepared by:

Dr. Laurent Couëtil, DVM, PhD

Professor, Veterinary Clinical Sciences

Director, Equine Sports Medicine Center

Director, Equine Research Programs

PURDUE UNIVERSITY

Equine Sports Medicine Center

INDEX

MISSION

To provide first class veterinary diagnostic and investigative support to the horse industry in Indiana and to educate owners, trainers, and veterinarians.

GOALS:

The goals of the ESMC are to pioneer leading-edge research in the area of equine sports medicine, to provide training to future equine veterinarians and veterinary technicians, to offer continuing education to Indiana veterinarians and horsemen, and to diagnose and treat causes of decreased performance in horses.

ACHIEVEMENTS OF EQUINE SPORTS MEDICINE CENTER (ESMC)

Treadmill Evaluations:

Treadmill diagnostic work-ups are an important activity at the ESMC. Five client-owned horses were evaluated on the treadmill in 2021. This brings the total number of horses evaluated since the opening of the ESMC in April 1996 to **543**. No demonstration was given in 2021 because of COVID-19.

Continuing Education and Extension Service:

- Continuing Education presentations:

  - Adams SB.

    Regional and State

    - Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
      - 40 years of equine surgery

  - Couetil L.

    International

    - Equine asthma treatments: Where is the evidence? *Boehringer Ingelheim Global splash event,* online. April 2021.

    National

    - What do we know about the pathophysiology of equine asthma? *The 17th American Association of Equine Practitioners Convention,* Nashville, TN, December 2021.
    - What every technician should know to help diagnose and manage asthma in horses. *American Association of Equine Veterinary Technicians,* Nashville, TN, December 2021.
    - Vet-PD Seminar on Equine Asthma, May 2021:
      - Diagnostic challenges: Mild-moderate vs. Severe Equine Asthma
      - Prevention and treatment of equine asthma

    Regional and State

    - Purdue University Back to Class Program, April 8th, 2021.
      - Even horses get asthma
    - Purdue Equine Science Club. Online presentation via Zoom, November, 2021.
      - Some lipids are good for horses' lungs

- Farr, A.

Regional and State

- Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
  - Horse-Proofing the Barn

- Hawkins, J.

Regional and State

- Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
  - Sinus and Guttural Pouch Disease

- Lescun, T.

Regional and State

- Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
  - Laminitis: What's new?
- Purdue Equine Science Club. Online presentation via Zoom, April, 2021.
  - Exciting equine science topics
- Purdue Equestrian Team. Online presentation via Zoom. April, 2021.
  - Diseases of the Horse Foot

- Taylor, S.

National

- Veterinary Meeting and Expo (North American Veterinary Conference), Orlando, FL, 2021.
  - Equine emergencies: case presentations
  - Assessment and management of critically ill neonatal foals
  - Medical emergencies of the equine abdomen
  - Equine botulism

Regional and State

- *Indiana Association of Equine Practitioners Annual Meeting*, 2021. Total of 6 lecture hours (virtual).
  - Theiler's disease: what's new?
  - Piroplasmosis: is it still a foreign animal disease in the U.S.?
  - Infectious enterocolitis
  - Equine gastric ulcer syndrome
  - Equine protozoal myeloencephalitis (EPM): an update
  - Management of botulism in an adult horse
  - Fever of unknown origin: emerging pathogen in the Midwest?
  - Tick paralysis in two American Miniature horses

- Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
  - When the filters fail: kidney and liver disease in horses.

- Tinkler, S.

Regional and State

- Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
  - Muscle Disease

- Waxman, S.

Regional and State

- Purdue Horseman's Forum, Purdue College of Veterinary Medicine, IN, February 6th, 2021.
  - Neck and Back Pain in the Horse

- Committee service

International

- Couetil, L:
  Dorothy Havemeyer Workshop on Equine Asthma – Chair of the organizing committee

- Tinkler, S:
  Vice President, Equitarian Initiative (Board of Directors 2016-present)

- Towsend, W:
  Research Committee Member, International Equine Ophthalmology Consortium. 2013-present

National

- Couetil, L:
  American College of Veterinary Internal Medicine
  - Board of Regents Nomination Committee
  - Board of Regents Governance Committee

- Kritchevsky, J:
  American Humane Society Scientific Advisory Committee for American Humane Farm Program
  Equine Endocrinology Working Group

- Lescun, T:
  American College of Veterinary Surgeons Foundation, Board of Trustees (2018-present)
  Veterinary Orthopedic Society, Research Committee (2021-present)

- Townsend, W:
  President, American College of Veterinary Ophthalmologists, 2020-21.
  Genetics Committee, American College of Veterinary Ophthalmologists, 2012-present
  Veterinary Ophthalmology, Editorial Board (2014 – present).

State:

- Lescun. T:
  Purdue Liaison to the Board of Directors, Indiana Association of Equine Practitioners 2016-present

***Outreach*:**

- Purdue's Equine Sports Medicine Center Web site dedicated to informing horse owners about equine-related activities at Purdue University has undergone a major update this year. The address of the site is: https://vet.purdue.edu/esmc/index.php

- Outreach activities

  - *Purdue Horseman's Forum*, Purdue Veterinary Medicine, West Lafayette, IN, February 6th, 2021.
    - Online Continuing Education meeting for horse owners and veterinarians
    - Approximately 200 registrations, 8 lectures

- Lay Publications*:*

  - The Equine Sports Medicine Center continued publication of its newsletter called "**Equine Health Update**" established as a source of information for Indiana's horse industry. Dr. Stacy Tinkler is the editor for the newsletter since January 2012. One issue was released in 2021 and articles are accessible from our Web site. The newsletter is included in Appendix A **(Blue)**.

  - Baird, T; Lescun, T:
    - Dorsal metacarpal disease of Thoroughbreds. Equine Health Update for Horse Owners and Veterinarians, Vol. 23, Issue No. 1, 2021.

  - Brink, L; Tinkler, S:
    - "The heart of the matter: Can a horse with a heart murmur still perform?" - Equine Health Update for Horse Owners and Veterinarians, Vol. 23, Issue No. 1, 2021.

  - Cipolla, S; Gillespie-Harmon, C:
    - The current state of physical rehabilitation in equine medicine - Equine Health Update for Horse Owners and Veterinarians, Vol. 23, Issue No. 1, 2021.

  - Couetil, L:
    - The Horse Magazine "Low-dust forages essential for asthmatic horses". 2021

  - Taylor, S.D., Olave, C:
    - Mysterious liver disease in Midwestern horses. Purdue University College of Veterinary Medicine Equine Health Update. 2021;23(1):3.

  - Townsend, W:
    - "The eyes have it: Tips on keeping your pet seeing clearly" in *Indiana REMC Magazine*, March 2021, page 30.
    -

  - Van Matre, A; Taylor, S.D:
    - "Clostridial enterotoxemia in neonatal foals". Purdue University College of Veterinary Medicine Equine Health Update. 2021;23(1):4.

***Research*:**

Research activities from investigators of the Equine Sports Medicine Center are summarized below. The names of members of the ESMC are underlined. Because of the impact of COVID-19 on research activities, no call for proposal was released FY-2021. Investigators with active research projects were granted 1-year, no-cost extensions.

Research projects in progress supported with Pari-Mutual Funds:

Progress reports for the following projects are included in Appendix B **(Gold).**

Dangoudoubiyam, S**. Effect of Ivermectin on the Release and Biomolecule Content of Extracellular Vesicles Derived from Parascaris spp. Larvae.**

Hooser, S. **Detection of Black Walnut Wood in Equine Bedding by PCR**.

Lescun, T., Hermida J., Main R.P., Little, D., Weng, H-Y. **Collagen Orientation and Tensile Strength of Equine Proximal Sesamoid Bones.**

Little, D., Lescun, T**. Mechanosensitive Channels in Equine Musculoskeletal Soft Tissues.**

Taylor, S.D., Kritchevsky. J.E., Olave, C., Miller, M. **Understanding Midwest Acute Cholangiohepatitis.**

Wilkes, R., Kattor, J. **Evaluation of Targeted Next Generation Sequencing for Detection of Equine Pathogens in Clinical Samples.**

Research projects completed supported with Pari-Mutual Funds:

Complete reports for the following projects are included in Appendix C **(Green)**.

Figueiredo, M., Lescun, T., Gimble, J. **Enhancing the Repair Potential of Equine-Derived MSC for Treating Post-Traumatic Osteoarthritis.**

Hendrix, K., Kritchevsk,y J**. Recovery of Salmonella Bacterial Isolates from Pooled Equine Fecal Samples.**

Taylor, S.D., Anderson, M., Reinhart, J., Cooper, B. **Pharmacokinetics of Thiamine Hydrochloride.**

Competitive Equine Research Fellowship supported with Pari-Mutual Funds:

The PVM Equine Research Fellowship is for the recruitment of outstanding M.S. or Ph.D. track students to conduct applied/clinical research in the area of equine medicine at Purdue University to address issues of importance to the health and performance of Indiana racehorses and other equine athletes. The fellowship provides one year (M.S.) or two years (Ph.D.) of stipend support from the PVM Equine Internal Fund and additional years of funding support for degree completion will come from the graduate program of the respective department.

Jesus Hermida, DVM, Third-year PhD student in VCS – Faculty advisors: Drs. Tim Lescun & Russell Main: *Application was approved by the ERAB and the Equine Research Fellowship was granted for the 2020-2021 academic year.*

Externally funded equine research projects conducted in 2020:

Couetil, L.L., Ivester, K.., Moore, G.., Olave, C., Park, J.H., Burgess, J., Mukhopahyay, A. Asthma, Performance and Omega-3s in Racing Thoroughbreds. Grayson Jockey-Club Research Foundation. $210,016.

Couetil, L.L., Olave, C., Ivester, K. Specialized Pro-Resolving Lipid Mediators in Equine Asthma. Boehringer Ingelheim Vetmedica. $15,000.

Couetil L.L., Ivester, K., Tyson, K. Efficacy of Nebulized Dexamethasone in Horses with Mild Airway Inflammation. Nortev Ltd. $75,593.

Couetil, L. Effects of Low-Dust Forage and Omega-3Ffatty Acids on Racehorses' Lung Health. USDA-NIFA (IND90000122-Hatch): 2020-2025.

Figueiredo, M., Sintim, Lescun, T.B. Developing Novel Laminin Receptor-Targeted Drugs to Promote Joint Repair in Equine Osteoarthritis. PVM Internal Competitive Research Funds Program. $25,000 (2020 to 2022).

Figueiredo, M., Sintim, Lescun, T.B. Developing Novel Laminin Receptor-Targeted Drugs to Promote Joint Repair in Equine Osteoarthritis. Purdue AgSEED. $50,000 (2020 to 2022).

Figueiredo, M. Developing Novel Laminin Receptor-Targeted Drugs to Promote Joint Repair in Equine Osteoarthritis. USDA-NIFA (IND0011848-Hatch): 2020-2024.

Hart, H., Barton, M., Hurley, D., Taylor, S.D. Metabolic Resuscitation in Equine Neonatal Sepsis: A Novel Therapeutic Strategy. Morris Animal Foundation. $147,390 (2019-2022).

Kritchevsky, J. Use of a Penetrating Captive Bolt as a Practice-Based Euthanasia Method in Horses. Center for Animal Welfare Science. $22,227.75.

Kritchevsky, J. Investigation of Genetic Risk Alleles for Pituitary Pars Intermedia Dysfunction. Morris Animal Foundation. $58,959.

Lescun, L., Hermida, J., Main, R., Little, D., Weng, H-Y. Collagen Fiber Orientation and Mechanical Properties of Equine Proximal Sesamoid Bones. ACVS Foundation. $21,000.

Lescun, T. Modeling Orthopedic Injuries of the Foot and Fetlock in the Horse. USDA-NIFA Hatch Project (IND020784): 2018-2023.

Schott, H., Taylor, S.D., Carr, E. Investigation of an Emerging Seasonal Hepatitis Syndrome in Horses. Michigan State University CVM. $59,693 (2021-2022).

Tinkler, S., Van Wormer, L., Varnum, A. Program Request for Peru 2019 Equitarian Project (5 Andean communities). Zoology Foundation. $25,000.

Tinkler ,S., Merma, O.A., Varnum, A. COVID-19 Relief and Response -Peru 2020 Equitarian Project. Zoology Foundation. $15,000.

Tinkler, S., Van Wormer L., Varnum, A. Peru 2021 Equitarian Project (5 Andean communities). AAEP Foundation. $5,000.

Tinkler, S., Van Wormer L., Varnum, A. Ecuador 2021 Equitarian Project Vet student education and capacity building – Quininde. AAEP Foundation. $5,000.

Tinkler, S., Van Wormer L., Varnum, A. Distance Education - Online Learning Platform Development. AAEP Foundation for the Horse. $30,000.

Witonsky, S., Taylor, S.D, Reed S, McKay R, Clark C, Furr M. Is soluble CD14 (sCD14) Increased in Specific Equine Neurologic Diseases? Virginia-Maryland Equine Research Competition Grant. PI: S. Witonsky; Co-I: S. Taylor, S. Reed, R. MacKay, C. Clark, M. Furr. $10,000 (2021-2022). Virginia-Maryland Equine Research Competition Grant. $10,000.

Publications supported by the Equine Research Internal Funds: Appendix D (Purple)

The names of members of the ESMC are underlined.

Refereed Scientific Articles:

Couetil, L.L., Ivester, K., Barnum, S., Pusterla, N. Equine Respiratory Viruses, Airway Inflammation and Performance in Thoroughbred Racehorses. *Vet Microbiol*. 2021 Jun;257:109070. doi: 10.1016/j.vetmic.2021.109070. Epub 2021 Apr 10.

Gray, .S, Gutierez-Nibeyro, S.D., Couëtil, L.L, Secor, E.J., Schaeffer, D.J. Evaluation of the Airway Mechanics of Modified Toggle Laryngoplasty Constructs using a Vacuum Chamber Airflow Model. *Vet Surg*. 2021 Jul 26. doi: 10.1111/vsu.13690. Epub ahead of print.

Hess, E.K., Reinhart, .JM., Anderson, M..J, Jannasch, A.S., Taylor, S.D. Pharmacokinetics of Thiamine (vitamin B1) in Adult Horses after Administration of Three Single Intravenous Doses. *J Vet Pharmacol Ther*. 2021 Nov;44(6):937-944. doi: 10.1111/jvp.13007. Epub 2021 Aug 18.

Kritchevsky, J.E., Olave, C., Tinkler, S.H., Tropf, M., Ivester, K., Forsythe, L., Couëtil, L.L. A Randomised, Controlled Trial to Determine the Effect of Levothyroxine on Standardbred Racehorses. *Equine Vet J*. 2021 Jun 8. doi: 10.1111/evj.13480. Epub ahead of print.

Olave, C.J., Ivester, K.M., Couëtil, L.L, Kritchevsky, .JE., Tinkler, S., Mukhopadhyay, A. Dust Exposure and Pulmonary Inflammation in Standardbred Racehorses Fed Dry Hay or Haylage: A Pilot Study. *Vet J*. 2021. May;271:105654. https://doi.org/10.1016/j.tvjl.2021.105654.

Olave, C.J., Ivester, K.M., Couëtil, L.L, Franco, J., Mukhopadhyay, A., Robinson, JP., Park, J.H. Effects of Forages, Dust Exposure and Pro-Resolving Lipids on Airway Inflammation in Horses. Am J Vet Res. Doi: 10.2460/ajvr.21.08.0126. Epub 2021 Nov 25.

Serpa, P.B.S., Woolcock, A., Taylor, S.D., Pires Dos Santos, A. Validation of a Flow Cytometric Assay to Detect Intraerythrocytic Reactive Oxygen Species in Horses. *Vet Clin Pathol*. 2021 Mar;50(1):20-27. doi: 10.1111/vcp.12976. Epub 2021 Mar 1.

Taylor, S.D., Ivester, K.M., Stewart, C., Horohov, D., Couëtil, L.L. The Effect of Lower Airway Inflammation on Inflammatory Cytokine Gene Expression in Bronchoalveolar Lavage Fluid and Whole Blood in Racing Thoroughbreds. *Vet Immunol Immunopathol*. 2021 Jul;237:110266. doi: 10.1016/j.vetimm.2021.110266. Epub 2021 May 9.

Taylor, S.D., Kritchevsky, J.E., Slovis, N.M., Wilkin,s P.A., Austin, S.M., Schott, H.C. Emerging Outbreak of Hepatitis in Midwestern Horses. Letter to Editor, *J Am Vet Med Assoc*. 2021;258(11):1183.

Taylor, S.D., Serpa, P.B.S., Santos, A.P., Hart, K.A., Vaughn, S.A., Moore, G.E., Mukhopadhyay, A., Page, A.E. Effects of Intravenous Administration of Peripheral Blood-Derived Mesenchymal Stromal Cells after Infusion of Lipopolysaccharide in Horses. *J Vet Intern Med*. Under Final Review, December 2021.

Abstracts and Proceedings

Chang, T., Akin, S., Couetil, L, Byung-Guk, J., Lee, C.H. Dual Regime Spray of Functional Nanomaterials for Electronic Textiles. Materials Research Society meeting, Hawaii, May 2022.

Christmann, U., Hancock, C., Emery, A., Poovey, J., Ortega Morales, S., Duncan, A., Page, A., Couetil, L., Morresey, P., Wood, P. Horses with Severe Asthma have an Altered Surfactant Ceramide Profile. *Proceedings of the Veterinary Comparative Respiratory Society Symposium, 2021,* online meeting.

Constable, P.D., Tinkler, S.H., Couetil, L., Angela, Demaree. Predictors of Blood pH and Plasma. Conference of Equine Exercise Physiology, Sweden, June 2022.

Couetil, L.L. What do We Know about the Pathophysiology of Equine Asthma? *Proceedings of the 67th American Association of Equine Practitioners Convention,* Nashville, TN. December 2021.

Couetil, L.L. What Every Technician Should Know to Help Diagnose and Manage Asthma in Horses *Proceedings of the American Association of Equine Veterinary Technicians,* Nashville, TN. December 2021.

Couetil, L.L., Olave, C.J., Ivester, K.M., Burgess, J., Park, J.H. The Role of Dust Exposure and Omega-3 Polyunsaturated Fatty Acids on Thoroughbred Racehorses' Airway Health: A Randomized, Controlled Trial. *Calgary International Equine Symposium*, 2021 (online presentation).

Christmann, U., Hancock, C., Emery, A., Poovey, J., Ortega Morales, S., Duncan, A., Page, A., Couetil, L., Morresey, P., Wood, P. Horses with Severe Asthma have an Altered Surfactant Ceramide Profile. *Proceedings of the Veterinary Comparative Respiratory Society Symposium, 2021,* online meeting.

Gray, S.M., *Gutierrez-Nibeyro, S.D., Horn, G.P., Kesler, R.M., Couëtil, L.L., McCoy, A.M., Stewart, M.C, Lascola, L.M., Schaeffer, D.J. Evaluation of the Airway Mechanics of Modified Toggle Laryngoplasty Constructs using a Vacuum Chamber Airflow Model. *European College of Veterinary Surgeons* 2021 (online presentation).

Burcham, G., Hooser, S. Botulism: Case Studies in Cattle, Horses and Poultry. *Proceedings of the 2021 Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians*.

Book Chapters:

Allbaugh, R.A., Townsend, W.M. Diseases of the Equine Vitreous and Retina. In: Gilger, BC. Ed. *Equine Ophthalmology*. 4th ed. Ames, IA: John Wiley & Sons. Submitted, May 2021.

Couëtil, L.L. Equine Asthma in Athletic Horses. In: Hinchcliff K, Kaneps A, Geor R, van Erck E, eds. *Equine Sports Medicine and Surgery*. 3rd ed. Oxford: Elsevier Science; 2021.

Lescun, T.B. In: Rubio-Martinez LM, Hendrickson DA, eds. *Complications in Equine Surgery*. John Wiley & Sons; January 2021.

- Splint bone fractures and removal.
- Fractures of the head.

Refereed Scientific Publications: [Appendix E **(Gray)**]

Anis, E., Ilha, MRS., Engiles, J.B., Wilkes, R.P. Evaluation of Targeted Next-Generation Sequencing for Detection of Equine Pathogens in Clinical Samples. *Journal of Veterinary Diagnostic Investigation*, in press, 2020.

Hay, A.N/, Wagner. B/, Leeth. C/M/, LeRoith. T/, Cecere. T.E., Lahmers, K.K., Andrews, F.M., Were, S.R., Johnson, A.L., Clark, C.K., Pusterla, N., Reed, S.M., Lindsay, D.S., Taylor, S.D., Estell, K.E., Furr, M., MacKay, R.J., Del Piero, F., *Witonsky, S.G. Horses Affected by EPM have Increased sCD14 Compared to Healthy Horses. *J Immunol Immunopathol*. 2021;242, DOI: 10.1016/j.vetimm.2021.110338.

Hohu, K.K., Lim, C.K., Adams, S.B., Heng, H.G., Ramos-Vara, J.A. Ultrasonographic and Computed Tomographic Features of Rice Bodies in an Arabian Horse with Atlantal Bursitis. Vet Radiol Ultrasound. 2020 Jan;61(1):E1-E5. doi: 10.1111/vru.12596.

Keenan, A.V., Townsend, W.M. Evaluation of Equine Corneal Disease using Ultrasound Biomicroscopy. *Vet Ophthalmol*. 2021;00:1-6. Published online 11 March 2021. https://doi.org/10.1111/vop.12881.

Lyons, V.N., Townsend, W.M., Moore, G.E., Liang, S. Commercial Amniotic Membrane Extract for Treatment of Corneal Ulcers in Adult Horses. *Equine Vet J*. 2021;00:1-9. Dec 15. doi: 10.1111/evj.13399.

Skelton, J.A., Hawkins, .JF., Rochat, M.C. Treatment of Shoulder Joint Luxation with Glenoid Ostectomy in a Miniature Donkey. *J Am Vet Med Assoc*. 2021 Nov 1;259(9):1043-1046. doi: 10.2460/javma.259.9.1043.

EQUINE RESEARCH ADVISORY BOARD
Membership 2020-21

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Dr. Laurent Couetil	Chair	VCS-Lynn G408	765-494-6808	couetill@purdue.edu
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Dr. Jan Hawkins	3	VCS-Lynn G416B	765-494-8563	hawkinsj@purdue.edu
Dr. Steve Hooser	1	CPB	765-494-6931	shooser1@purdue.edu
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TBA		Indiana Thoroughbred Owners & Breeders Assoc.		

EQUINE RESEARCH ADVISORY BOARD
Membership 2021-22

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APPENDIX A

- ***Equine Health Update*** **- Equine Sports Medicine Center Newsletter Vol. 23, Issue No. 1 – 202*1***

PURDUE UNIVERSITY
College of Veterinary Medicine

EQUINE HEALTH UPDATE

FOR HORSE OWNERS AND VETERINARIANS

The Heart of the Matter:
CAN A HORSE WITH A HEART MURMUR STILL PERFORM?

By Laura Brink, DVM Student (Class of 2021)
Edited by Dr. Stacy H. Tinkler, Large Animal Internal Medicine

Finding out that your horse has a heart murmur may come as a surprise, especially if your companion has no outward signs of heart disease. The good news is that many horses are able to continue their current lifestyle without negative effects; however, moderate to severe murmurs due to underlying cardiac disease may have potentially devastating consequences.

The Healthy Equine Heart

A horse's heart consists of four chambers and four valves through which blood flows in an organized fashion (**Figure 1**). Oxygen-poor blood from the body enters the right atrium, flows through the tricuspid valve, and into the right ventricle. It then passes through the pulmonary valve into the pulmonary arteries where it becomes oxygen-rich in the lungs. From the lungs, blood flows through the pulmonary veins into the left atrium, then through the mitral valve into the left ventricle. Finally, blood passes through the aortic valve into the aorta for distribution to the body. Coordinated timing of valve closure and opening allows for efficient blood flow.

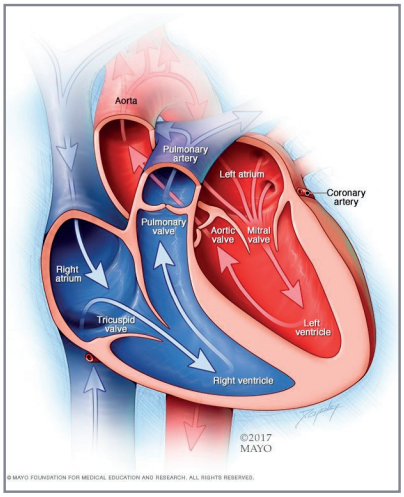

***Figure 1.** Normal heart*

Equine Heart Murmurs

The usual "lub-dub" of the heart beat is due to synchronized closure of the mitral and tricuspid valves ("lub"), then the synchronized closure of the pulmonary and aortic valves ("dub"). If a valve fails to open or close appropriately, another sound may be heard that is due to abnormal blood flow within the heart. This other sound is referred to as a heart murmur. There are two types of heart murmurs—physiologic—where the murmur is due to turbulent blood flow through a normal heart or pathologic—where the murmur is caused by a cardiac or valve defect. In order to determine the possible causes of a murmur, it

(continued on page 2)

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Heart Murmur *(continued from cover)*

must be characterized by its location, timing, and intensity and will be assigned a grade from 1-6 by your veterinarian, based on its intensity and loudness. It is important to note that a higher grade does not always mean the cause of the murmur is more severe.

In adult horses, two of the most common pathologic heart murmurs are due to aortic insufficiency (AI), or aortic valve regurgitation, and mitral insufficiency (MI), or mitral regurgitation. They are commonly caused by valve degeneration, in which the affected valve(s) cannot close properly, leading to backflow of blood and an audible heart murmur (**Figures 2 and 3**). Valvular degeneration is considered, in part, to be a normal aging change, though other underlying processes may also play a role. For example, severe MI can also be caused by inflammation from infection and may also arise secondary to chamber dilation due to progressive AI, or as a result of a birth defect.

Aortic valve regurgitation (AI) occurs most often in horses 10 years of age or older and studies have shown that male horses are more likely to develop AI than females. Generally, AI is a slowly progressive disease, meaning it worsens slowly over time. It often presents mildly with the majority of horses experiencing few to no changes in performance. Similarly, mitral valve regurgitation (MI) occurs most often in horses 15 years of age or older. MI progresses and presents in a similar fashion to AI, with slow progression and minimal impact on performance.

In cases of moderate to severe AI or MI, the changes in blood flow and blood volume within the different chambers of the heart can be detrimental. Moderate to severe AI causes the left ventricle to dilate (stretch in size), making it difficult to pump blood out of the heart. This can lead to cardiac arrhythmias (abnormal rhythms) and subsequent heart failure, which may be noticed by owners as intolerance of normal exercise, difficulty breathing, cough, edema, lethargy, and clear nasal discharge. Sudden death from arrhythmia may also occur. Moderate to severe MI leads to dilation of the left atrium and is poorly tolerated with horses more prone to developing arrhythmias and/or heart failure than those with AI alone.

Moving Forward—Work-Up, Management, and Prognosis

Once a murmur is identified, the goal is to determine the source and the severity of any underlying disease. To do this, an echocardiogram (ultrasound of the heart) will be performed to look at the heart's structure and function, as well as an electrocardiogram (ECG) to evaluate the heart's rhythm. Additional tests may be recommended depending on the patient.

Management of pathophysiologic heart murmurs is targeted at monitoring for worsening of disease. Yearly or twice-yearly exams with echocardiogram and ECG is recommended depending on severity of disease. In horses that develop congestive heart failure, medical therapy can be considered.

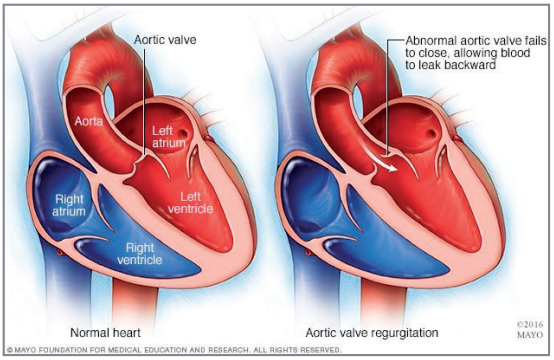
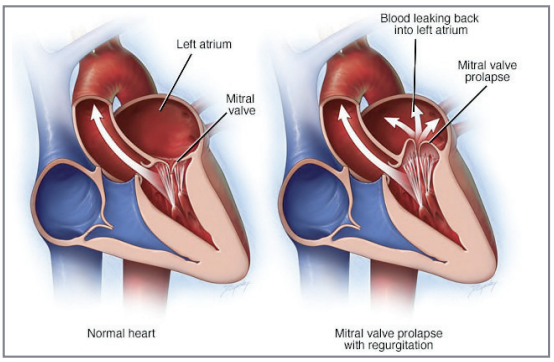


***Figure 2.** Aortic insufficiency (AI)*

***Figure 3.** Mitral insufficiency (MI)*



Overall, the decision whether or not a horse with a heart murmur can continue its current lifestyle is patient dependent. Patient safety, health, and rider/driver safety must all be considered prior to making this decision. Most horses with mild AI or MI can continue their normal lifestyle; however, horses with severe disease, heart failure, arrhythmias or heart chamber dilation should not be ridden due to welfare concerns for the horse and for rider safety due to the risk for sudden death.

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MYSTERIOUS LIVER DISEASE IN MIDWESTERN HORSES

By Drs. Sandra Taylor and Carla Olave, Large Animal Internal Medicine

In the autumn of 2020, sporadic cases of equine hepatitis began surfacing in several Midwestern states. The first symptoms were high fevers (up to 107°F!) and decreased appetite, with most affected horses then developing jaundice (yellowing of the gums and whites of the eyes; **Figure 1**). Blood work pointed to an inflammatory process with increases in liver enzyme activity. Fevers typically responded well to non-steroidal anti-inflammatory drugs, such as flunixin meglumine (Banamine®), but a second cycle of fevers was observed 1 - 2 weeks after the first cycle had ended. In a few horses, a third fever cycle was noted.

Initially, veterinarians suspected that these were cases of infectious gastrointestinal (GI) disease with secondary liver involvement (i.e. compression of the bile duct by a large, inflamed colon or backflow of intestinal bacteria up the bile duct into the liver),[1] but GI tests were normal. Stumped, veterinary internal medicine specialists (internists) at Purdue University began testing for everything under the sun, including COVID-19 (SARS-Cov-2)! Luckily, testing for COVID-19, anaplasmosis, leptospirosis, equine hepatitis viruses (equine parvovirus-hepatitis and equine hepacivirus), pathogenic equine respiratory viruses, Strangles, and pathogenic GI bacteria and viruses were negative. Although the livers of affected horses appeared normal on ultrasound examinations, liver biopsies showed significant liver inflammation (hence, the diagnosis of "hepatitis"). Some horses also had areas of necrosis (dead tissue) within the liver. Given the appearance of several types of inflammatory cells, it was difficult to know what type of insult the livers had suffered: bacterial? viral? fungal? toxic? To try to answer that question, liver and blood samples from affected horses were sent to laboratories throughout the country for unique, highly sophisticated molecular diagnostic testing in an effort to identify possible new pathogens. To date, no cause for this disease has been identified; efforts are ongoing.[2]

The good news? No affected horses that were presented to Purdue University with this idiopathic hepatitis syndrome developed liver failure. Also, all horses recovered clinically within 6 weeks of the first spike in rectal temperature and returned to their previous level of work. Antibiotics did not appear to be necessary for clinical recovery. However, follow-up liver biopsies in affected horses have shown improved but ongoing inflammation, even 3 - 4 months after clinical resolution.

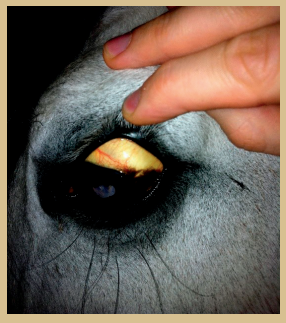

Figure 1. Jaundice (icterus) of the sclera in a horse.

It is unknown how long complete healing will take, and whether or not the liver will be scarred in the future. The other silver lining is that no new cases have been reported since April of 2021.

So what's going on? Purdue University pathologists and collaborating researchers around the country have been instrumental in getting to the bottom of this new syndrome. Although the cause has not been identified, several important diseases have been ruled out, including Theiler's disease (equine serum hepatitis), which has recently been discovered to have an association with equine parvovirus-hepatitis.[3,4] According to pathologists, it is unlikely that a toxin is involved given the microscopic appearance of the liver biopsies. Therefore, an infectious agent is most likely, and if this is true, it is unknown whether this agent is contagious to other horses. The majority of affected horses were in contact with unaffected horses, but a few farms reported more than one horse being affected. In addition, it is unknown whether or not new cases will be seen this coming autumn or winter. Perhaps this novel syndrome is seasonal, or maybe there was bacterial or viral contamination of an equine product. Importantly, epidemiological surveys have not identified a common thread among affected horses.

In summary, a new, apparently transient, hepatitis syndrome has been identified in Midwestern horses, the etiology of which is still unknown. Although we hope to never encounter this disease again, if it does return, additional blood and liver testing will be important in identifying a cause and helping determine long-term prognosis. As with any horse that demonstrates a decreased appetite and/or fever, please call your veterinarian immediately if you notice these signs. Do not administer anti-inflammatory medications (such as Banamine® or phenylbutazone/Bute) unless advised by your veterinarian. If this novel hepatitis syndrome recurs in previously affected horses or if new cases are detected, funds are available for testing. Fingers crossed that we won't need to use them, but please call Dr. Taylor in the Large Animal Clinic if you suspect that your horse has this condition or if you have any questions. Liver biopsies are performed under standing sedation and pose very little risk in most cases.

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Clostridial Enterotoxemia in Neonatal Foals

By Alicia Van Matre, DVM Student (Class of 2021) – Edited by Dr. Sandra Taylor, Large Animal Internal Medicine

There are many organisms that cause diarrhea in neonatal foals including *Salmonella* spp, *Rotavirus*, *Cryptosporidium parvum*, and *Clostridium* spp. Of these organisms, the clostridial bacteria result in the highest morbidity (illness) and mortality. In particular, the species to be aware of are *Clostridium perfringens* and *Clostridium difficile*. These bacteria cause disease through their ability to produce enzymes called exotoxins. An exotoxin is a protein produced from a live bacterium that is excreted into its surroundings, causing tissue damage. An endotoxin, by comparison, is a protein released when a bacterium is lysed (ruptured), and is also toxic to the surrounding environment. Endotoxins are primarily produced by Gram-negative bacteria, while exotoxins can be produced by Gram-positive bacteria, such as *Clostridium* spp, or Gram-negative bacteria.

Which toxins do *C. perfringens* and *C. difficile* produce?

C. perfringens and *C. difficile* can each produce several exotoxins depending on the bacterial strain. Regarding *C. perfringens*, researchers have divided the types of exotoxins into two categories: major and minor toxins. Major toxins are the exotoxins that research has proven to be lethal in mice. Minor toxins, on the other hand, still cause disease but are considered non-lethal. The major toxins include alpha, beta, epsilon, and iota and the strains of *C. perfringens* are classified based on which of these toxins they produce. Typically, Types A through E are the most common, but Type F and Type G have also been identified. The types that are most concerning in neonatal foals are *C. perfringens* Type A and Type C, due to the severity of disease they cause. *C. difficile*, by comparison, produces fewer exotoxins, two of which have been most associated with disease in foals. These toxins are Toxin A and Toxin B. They are similar in structure but are categorized differently: Toxin A is considered an enterotoxin and results in hemorrhage and damage to the mucosal lining of the intestines while Toxin B is a cytotoxin and it damages individual cells. Foals can become sick from either one or both toxins.

How do *C. perfringens* and *C. difficile* cause disease and what are the signs?

For both species of *Clostridium*, the bacteria enter the foal's body through ingestion. The most common source of *C. perfringens* is the dam; mares can shed the bacteria in their feces. When the newborn foal suckles or noses around its stall or paddock, they can ingest this bacteria. In some foals, the bacteria can produce toxins that cause inflammation of the intestines, which is termed "enterocolitis" (**Figure 1**). *C. difficile* works in a similar fashion: the foal ingests the bacteria from the environment and Toxins A and/or B can cause inflammation of the intestines. One important reason that neonatal foals are at risk for developing clostridial enterocolitis is because colostrum contains proteins that block an enzyme that can break down clostridial toxins. Therefore, most foals that develop clostridial enterocolitis have adequate passive transfer of colostral immunity.

***Figure 1.** Necrotic and hemorrhagic small intestine in a foal with clostridial enterocolitis. (Uzal, Vet Micro, 2012)*

Clinical signs of clostridial enterocolitis vary from mildly soft feces to severe watery and hemorrhagic diarrhea. Abdominal bloat is common since the bacteria can produce gas; this can cause colic and even intestinal rupture. Foals become dehydrated secondary to anorexia and diarrhea. In foals with enterocolitis, inflamed and damaged intestine can allow absorption of *Clostridium* spp. and normal intestinal flora from the gut into the blood. The presence of bacteria and toxins in the blood is termed "septicemia;" bacteria can then travel to joints, bones and the central nervous system and cause multiple organ damage.

Although clostridial bacteria can cause enterocolitis and septicemia in neonatal foals, not all foals that ingest *Clostridium* spp. get sick. In fact, *C. perfringens* Type A and *C. difficile* make up part of the normal bacterial flora of the foal's gut and usually do not cause disease unless the bacterial strain is particularly virulent or a large amount of bacteria is ingested. In addition, foals with concurrent illness are at higher risk of developing enterocolitis from clostridial bacteria because of a weakened immune system. Conversely, C. *perfringens* Type C is never considered part of the normal flora of the foal's gut. Type C typically causes more serious disease than Type A or *C. difficile* because Type C produces the *C. perfringens* beta (CPB) exotoxin. CPB is a necrotizing toxin, meaning it forms holes in the membranes of the cells it contacts, causing the death of those cells and the surrounding tissue.

Treatment and prognosis

Treatment of clostridial enterocolitis depends on severity of illness but often requires hospitalization and intensive care. Fluid and electrolyte therapy is typically required to correct dehydration and electrolyte loss from diarrhea. Antibiotic therapy is necessary in septicemic foals and is often given to specifically kill clostridial bacteria. Other treatments include the clostridial toxin binder, di-tri-octahedral smectite (Biosponge™), as well as lactase enzyme to aid in digestion. Plasma might be necessary to replace blood protein that is lost in diarrhea and to improve blood pressure in some cases. In severe cases, parenteral (intravenous) nutrition might be required while the gut heals.

The survival rate of foals infected with *C. perfringens* Type C is 50% compared to 70% of those infected with *C. perfringens* Type A and *C. difficile*. The severity and rapid progression of these infections is why it is imperative that these foals are assessed by a veterinarian at the onset of clinical signs. Aggressive treatment is critical for a positive outcome.

Can clostridial enterocolitis be prevented?

Clostridial infections usually develop within the first week of life. Therefore, all foals must be watched carefully over the first several days following birth, even if the foal nursed colostrum well. Unfortunately, there are no specific protocols available that can guarantee the prevention of a clostridial infection. The bacterial spores are resistant to many disinfectants and can survive in the

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THE CURRENT STATE OF

Physical Rehabilitation in Equine Medicine

Co-authored by Shelby Cipolla, DVM Student (Class of 2021) and Dr. Caroline Gillespie Harmon, Equine Field Service

Physical rehabilitation has a plethora of potential applications within equine medicine—including post-operative recovery, following an injury or period of decreased activity, neurologic conditions, and improving strength and balance in a patient. In fact, there are few horses who would not benefit from some form of rehabilitation, whether fit or debilitated. The many varied goals of veterinary rehabilitation are to reduce pain, enhance tissue healing, improve endurance and muscle strength and ultimately to restore the animal to its previous activity level all while trying to prevent additional injury.[1] The field of equine rehabilitation includes the following modalities: hydrotherapy, dynamic mobilization (stretching), acupuncture, structured exercise programs, extracorporeal shockwave therapy, therapeutic laser treatment, and many others.[2] This article seeks to review the possible indications and benefits of some of the physical rehabilitation methods currently available.

One of the most frequently utilized rehabilitation methods is hydrotherapy (applying water to encourage healing). There are four types of equine hydrotherapy: swimming—which can help horses rehabilitate after injuries such as ligament damage, water treadmills—which can improve a horse's range of motion, equine spas—where the cooler water temperatures can decrease inflammation, and cold hosing. Cold water hydrotherapy has a wide range of indications including but not limited to wounds, laminitis (acute and chronic), and cellulitis.[3] Responses to this treatment include benefits such as decreased inflammation, pain reduction, locally restricted blood flow and decreased local metabolism. Alternatively, hot water hydrotherapy is indicated in situations where increased tissue stretching is required and in injuries that are at least 72 hours old. Therapeutic results include an increase in local blood flow and local metabolism, reduction of muscle spasms, increased extensibility of local tissues, and decreased pain.[4]

Another therapeutic modality commonly performed is dynamic mobilization, or more simply "carrot stretches." Appropriately named, "carrot stretches" involve luring the equine patient into a particular position with a treat and holding the position for a predetermined amount of time. Beneficial to almost any horse, these stretches focus on strengthening and balancing the core muscles while also increasing flexibility. They have been proven to increase the size of the muscles most influential in stabilizing the spine, the multifidus muscles.[4] These stretches can be used to help prepare young horses to be trained under saddle, older horses to maintain strength and flexibility, athletic horses to increase their performance capabilities, and following injury or surgery to build up strength prior to a gradual return to work.[5] An additional benefit of dynamic mobilization is that there are many stretches that can be used by owners at their home stable.

Acupuncture incorporates the insertion of fine needles through the skin in specific spots identified as treatment locations (acupoints). These are typically associated with a significant response from the horse through a variety of neurologic mechanisms and actions as well as connective tissue and fascial mechanisms that are beyond the scope of this article. When placed, the needles are often twisted and allow for the tissue to grip them and then stretch. This produces relaxation of the tissues and release of an analgesic. Acupuncture is a valuable tool in multi-modal therapy by helping quiet down triggered areas and allowing other treatments to work, but it is still important to find the primary underlying cause of pain in any patient if you want resolution of the ongoing problem.[1]

Dr. Jennifer Koziol performing acupuncture on a horse.

Exercise programs are utilized in order to gain muscle, tendon or ligament strength, or flexibility. They can include everything from hand walking, jogging in hand, walking under saddle, walking over cavalettis or up and down hills, and slowly increasing the amount or duration of activity over time. Horses are often started back to work after an injury slowly with some combination of the above regimens. This allows for the tissues to gain strength while preventing further damage to the healing tissue. Your veterinarian may ask you to return them to their normal work load over the course of one to two months or over as long as a year. Occasionally a treadmill is utilized as a method of providing exercise in a very controlled manner.

A patient undergoing shockwave treatment for a severely damaged ligament.

Extracorporeal shockwave is when pressure waves are generated and focused to then penetrate the tissue in question. This ultimately leads to micro-trauma which increases blood flow and nutrients to the desired area. Shockwave also provides some pain control. This is most prominent at 48 hours after treatment and is important because your horse may feel better and injure tissue that they are not protecting. Shockwave treatments are usually performed every 2-3 weeks and are typically used for tendon and ligament issues but it certainly can be utilized for back pain.[6]

Another commonly utilized rehabilitation method is laser therapy. This includes using a therapeutic low-level laser on a wounded or painful area in order to reduce inflammation and pain, increase endorphins, and stimulate healing (depending on the dose used).

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News & Notes

MEET OUR TREATMENT TECHNICIANS

My name is Allison Bayless. I am a registered veterinarian technician (RVT) at Purdue University Large Animal Hospital! I am a treatment technician and surgery on-call technician. I enjoy teaching fourth year veterinary students on their clinical rotations, interacting with clients, and caring for patients. Continually learning and meeting new people at Purdue makes for a valuable experience. I enjoy showing goats, judging livestock shows, traveling, hiking and kayaking in my free time.

My name is Kim Whitcomb and I am an RVT (registered veterinary technician) that works as one of the members of the Large Animal Treatment Team. I graduated from Purdue's Veterinary Nursing Program in 2019. Upon graduation I started working at Purdue as a treatment tech. Purdue has definitely allowed me to grow into a better technician and in the process I have learned so much. I love how I get to do so much with the patients and being able to help the students grow has been rewarding as well. In my free time I love to play softball, walks with my Golden Retriever, and hanging out with friends and family.

Clostridial Enterotoxemia

(continued from page 4)

environment for extended periods of time. Good biosecurity and hygiene practices are the most important components of prevention. Simple measures such as cleaning stalls regularly to remove manure that could potentially contain clostridial bacteria can reduce the amount of bacteria in the environment and reduce the likelihood of ingestion. Studies have suggested that washing the mare's udder following foaling to ensure that the teats are clean when the foal suckles can also help prevent infection. Thoroughly disinfecting foaling stalls between use, and having strict isolation protocols between mare and foal pairs is key in reducing the risk of spreading infection and preventing farm outbreaks of clostridial infections.

Another method to decrease the risk of *C. perfringens* infection is to vaccinate the pregnant mare with a *C. perfringens* toxoid at 6 and 3 weeks prior to foaling. The idea is to have antibodies against the *C. perfringens* bacteria present in the colostrum when the foal is born. There is unfortunately no vaccine for *C. difficile* at this time. Although it is tempting to prophylactically treat foals with antibiotics in an effort to prevent clostridial enterocolitis, research has shown that *C. difficile* infections can be caused by administration of antibiotics and some strains of *Clostridium* spp. are showing antibiotic resistance; therefore, **prophylactic antibiotic use is not recommended** to prevent these infections. Consulting your veterinarian to establish preventative measures is highly recommended.

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Rehab

(continued from page 5)

Common indications for laser therapy include osteoarthritis, wounds, soft tissue injuries, and localized pain.[3] Studies have reported that lasers provide pain relief as well as have a positive effect on wound healing. Laser treatments are performed multiple times a week.

It is well understood that physical rehabilitation is a valuable and versatile component of equine medicine. It would be rare to find a horse that could not benefit in some way from what it can offer; however, extensive research has not yet been performed in this field. There are very limited numbers of well-designed, blinded, randomized, controlled studies, cohort studies, or systematic reviews on the topic of equine physical rehabilitation.[7] Many of the specific techniques in equine physical rehabilitation, even some of the most frequently utilized such as those described in this article, are chosen based on "unsubstantiated claims of effectiveness or indications for treatment", as well as clinician experience.[8] There is an urgent need for well-designed studies in almost all the elements of equine physical rehabilitation so keep this in mind as you incorporate them into any programs for your equine companions.

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Dorsal Metacarpal Disease of Thoroughbreds

By Taylor Baird, DVM Student (Class of 2021) – Edited Dr. Tim Lescun, Large Animal Surgery

Dorsal metacarpal disease (DMD) is one of the most common performance-limiting conditions of racehorses. This disease complex consists of a painful periostitis and potentially stress fractures of the dorsal surface of the third metacarpal bone (MC3) in horses. This injury primarily affects Thoroughbreds but has been seen in Standardbreds and Quarter Horses as well. Clinical signs of DMD include pain with digital palpation and swelling on the dorsal, and sometimes dorsomedial, aspect of MC3 and is seen in mostly 18 to 36 month-old Thoroughbred racehorses.[1] In severe cases, these changes can be accompanied by macroscopic bony enlargements that can change the shape and contour of the bone, giving this disease the nickname "bucked shins." Studies show incidence as high as 70% in racehorses in the US, making this disease arguably one of the most common causes of lost training days and racing in Thoroughbred racehorses and the subject of much investigation.[2]

Over the years, racehorses have been asked to run harder and faster, leading to an increased muscle mass and an intensive training schedule at a young age. This intensive regimen at a younger age combined with a heavier horse has led to vulnerabilities in the limbs and reduced ability of MC3 to adapt quickly. When young horses are put into training during this time, their bone is introduced to high levels of stress. According to Wolff's law, training and racing induces an increase in thickness of the bone in order to withstand the strain placed on it. If repetitive, high-speed, cyclic exercise is introduced before the bone can adapt adequately, damage in the form of microfractures can occur. In response to this type of fatigue damage, excessive periosteal bone growth occurs. At around 2 years of age, MC3 has a larger amount of resorption cavities and incompletely filled secondary osteons than older horses due to the resorption of the primary osteons. This makes for a more porous, less stiff bone that is even more susceptible to microdamage. Greater strain on the bone results in more movement of the dorsal cortex during high-speed exercise. Since primary bone is stronger than remodeled bone, horses with DMD are susceptible to future fracture that could be detrimental to their careers and health.[1]

While DMD has a few different manifestations, they all arise from the same cause—intensive training before the horse is skeletally adapted to the training loads. This has been shown in multiple studies demonstrating the increase in bone density of horses undergoing exercise. This greater strain can lead to low cycle fatigue of the bone resulting in bone pain and even fracture. This is seen even more so in the lead limb of racehorses (the left limb in the United States) and is the location where fractures are more likely to occur in DMD.[3]

Treatment of DMD depends on the severity and chronicity of the disease. For most horses with acute DMD, 5 to 10 days of rest and NSAIDs are adequate to return them to light training. This training must be gradually increased with constant monitoring of the dorsal surface of MC3. An ease back into training allows MC3 to model according to the stress demands without producing structural damage. For subacute and chronic cases, the amount of rest needed to return to pain-free training is about 110 days.[1] There are several surgical options for treatment of dorsal MC3 fractures that include placement of a neutral unicortical screw in a lag fashion and/or dorsal cortical drilling (osteostixis). A study from California used records of 116 horses with dorsal cortical fractures of MC3 that were repaired with lag screw fixation to show the successful return to racing potential after surgery. In this study, all horses had radiographic resolution of the fracture 60 days after surgery and only 7% of the fractures reoccurred.[4]

Extracorporeal shock wave therapy is another treatment modality available. These shock waves are acoustic pressure gradient waves that are utilized to treat a variety of musculoskeletal diseases in both humans and animals. While the exact mechanism of how shock wave therapy exerts its healing effects on tissues is unknown, pain relief over the treated areas has been documented.[5] For this reason, it is important not to exercise performance horses with predisposing lesions (like DMD) too soon after the application of shock wave therapy. A study done in New Jersey demonstrated the added benefit of radial shock wave therapy when combined with an appropriate training program in the treatment of DMD. In this study, 45 of 50 horses diagnosed with DMD that was unresponsive to conventional therapy were able to return to racing.[6]

Thankfully the prognosis for DMD is very good. Advances in modern medicine like gait analysis and nuclear medicine have led to more information on DMD, and will hopefully provide more insight into predisposing factors in the future and an ability to prevent the disease in most cases.

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EQUINE HEALTH UPDATE

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The Equine Sports Medicine Center

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APPENDIX B

Research Projects in Progress Supported with Pari-Mutual Funds:

- Dangoudoubiyam, S. **Effect of Ivermectin on the Release and Biomolecule Content of Extracellular Vesicles Derived from Parascaris spp. Larvae.**

- Hooser, S. **Dectection of Black Walnut Wood in Equine Bedding by PCR.**

- Lescun, T., Hermida, J., Main, R.P., Little, D., Weng, H-Y. **Collagen Orientation and Tensile Strength of Equine Proximal Sesamoid Bones.**

- Little, D., Lescun, T. **Mechanosensitive Channels in Equine Musculoskeletal Soft Tissues.**

- Taylor, S.D., Kritchevsky, J.E., Olave, C., Miller, M. **Understanding Midwest Acute Cholangiohepatitis.**

- Wilkes, R., Kattor, J. **Evaluation of Targeted Next Generation Sequencing for Detection of Equine Pathogens in Clinical Samples.**

Pilot Equine Grant Program – Progress report

December 14th 2020

Sriveny Dangoudoubiyam

Research proposal title: Effect of ivermectin on the release and biomolecule content of extracellular vesicles derived from *Parascaris* spp. larvae.

Grant period: April1st, 2020 – March 31st, 2021

Grant money: $5000

Objectives of research: Parasite-derived extracellular vesicles play an important role in host-parasite interaction. The proposed research project had aimed at characterizing the changes induced by ivermectin on the protein and lipid content of the equine ascarid, *Parascaris* spp larvae-derived extracellular vesicles (EVs). Comparison of the biomolecule content of EVs from ivermectin-treated and ivermectin-not treated *Parascaris* larvae groups will facilitate identification of potential biomarkers for detecting and/or monitoring resistance to the anthelmintic, ivermectin.

The first phase of the project would involve collection of *Parascaris* adult females and/or *Parascaris* eggs, embryonation and hatching of larvae to establish in-vitro cultures of *Parascaris* larvae. The EVs will be isolated from the larvae-grown culture medium.

The second phase of the project would involve proteomic and lipidomic analysis of the EVs from both groups to identify potential biomarkers of ivermectin resistance.

Progress Summary and Challenges: Our project is in the first phase wherein we are collecting *Parascaris* adult female worms (from necropsied equine) and *Parascaris* eggs from equine fecal samples. Covid-related circumstances had posed a significant impediment to research progress in terms of acquiring samples, arrival of graduate students and functioning of the research laboratory in general. The adult female *Parascaris* worms were being collected from the research herd maintained at Gluck Equine Research Center. The worms recovered from this herd, this year were not favorable to our project, as we ended up having fewer adult females and a majority of male and immature worms. Nevertheless, the adult female worms and eggs collected so far have been appropriately preserved to perform embryonation and hatching.

Future activities:

1. We will continue to collect adult female *Parascaris* worms and eggs from horse fecal samples.
2. Embryonation, hatching and in-vitro larval cultures will be established to isolate extracellular vesicles from the culture medium.
3. Proteomic and lipidomic analysis will be performed.

Graduate students in my laboratory are now expected to start their research program in January 2021 and therefore, I expect to see the project make progress.

Request for extension: Due to unexpected hurdles that have hampered the progress of this research project, I humbly request the committee to grant a one-year, no-cost extension beyond the end date.

Detection of Black Walnut Wood in Equine Bedding by PCR

Final Report

30 April 2020

Summary: The goal of this project was to develop a PCR-based test to identify black walnut wood in bedding used for horses, with the aim to make the test available to ADDL clients. The PCR method described below does positively identify black walnut wood with high specificity and sensitivity. The method will now be adapted for use in the routine Molecular Diagnostics workflow and following further validation using bedding from diagnostic cases, this method should be ready for routine use by ADDL clients.

Hypothesis: Black walnut (*Juglans nigra*) wood can be identified in wood products used for equine bedding material ranging from sawdust to wood shavings using the polymerase chain reaction (PCR).

Specific Aim 1, to design and validate a method of sample preparation for black walnut wood detection using PCR, including determinations of the sensitivity and specificity of the method, has been completed (see attached abstract presented at the annual meeting of the American Association of Veterinary Laboratory Diagnosticians, October 27, 2019).

Specific Aim 2, to adapt the method (SA1) for the ADDL Molecular Diagnostics testing platform and perform in-house validation, has been largely completed. The method from SA1 is being adapted to the ADDL testing platform. To ease sample preparation and increase DNA yield, a commercial plant DNA extraction kit will be compared to the current DNA extraction method. Validation using stored samples from historical field cases in which horses were affected with clinical signs indicating exposure to black walnut wood, will then be completed.

Specific Aim 3 is to make the test available to ADDL clients and demonstrate the method on samples from field cases. Further validation using samples from contemporary field cases submitted to the ADDL will be carried out as they arrive and are analyzed.

Work to be Completed: Following the DNA extraction kit comparison & selection, and test validation using clinical samples in SA2, the test will be made available to ADDL clients. We anticipate that the commercial kit will work satisfactorily and that clinical samples will be positive since the black walnut DNA should be stable in our case samples. Validation of new tests using field samples is required as a part of the ADDL Quality System. Until a sufficient number of samples from field cases have been tested, a disclaimer will be added to final case reports stating that the test is still in the validation process. We anticipate being able to offer the test in 2020 at a time following easing of COVID19 restrictions.

Future Studies: The Equine Research Advisory Board has granted a no-cost extension until March, 2021 to continue development of diagnostic PCR-based tests for other poisonous plants of interest to Indiana horse owners.

FINAL / PROGRESS REPORT for COMPETITIVE EQUINE RESEARCH FUNDS, 2021

March 31st, 2021

TITLE OF GRANT: Collagen orientation and tensile strength of equine proximal sesamoid bones

PI: Timothy B. Lescun; **Co-Investigators:** Jesus Hermida, Russell Main, Dianne Little, Hsin-Yi Weng

This ex-vivo experiment was designed to investigate the relationship between regional collagen fiber orientation (CFO), tensile mechanical properties and mineralized microstructural features of proximal sesamoid bone (PSB), in both horses who suffered a fracture of this bone and non-fracture control horses from samples collected over 5 years in a single racetrack in the state of Indiana.

The **specific aims** are:

1. To determine whether longitudinal CFO in the PSB is lower in horses with fracture of this bone compared to non-fracture control horses.
2. To compare the regional tensile mechanical properties, mineralized microstructural features and water content of the contralateral PSB from horses with a PSB fracture to non-fracture control horses.

Forelimb PSBs from twenty horses, ten euthanized due to PSB fracture (fracture horses) and ten euthanized for reasons other than a musculoskeletal injury (control horses) from horses obtained from the Indiana Horse Racing Commission necropsy program at the Indiana Animal Disease Diagnostic Laboratory at Purdue University were identified for this study. Figure 1.

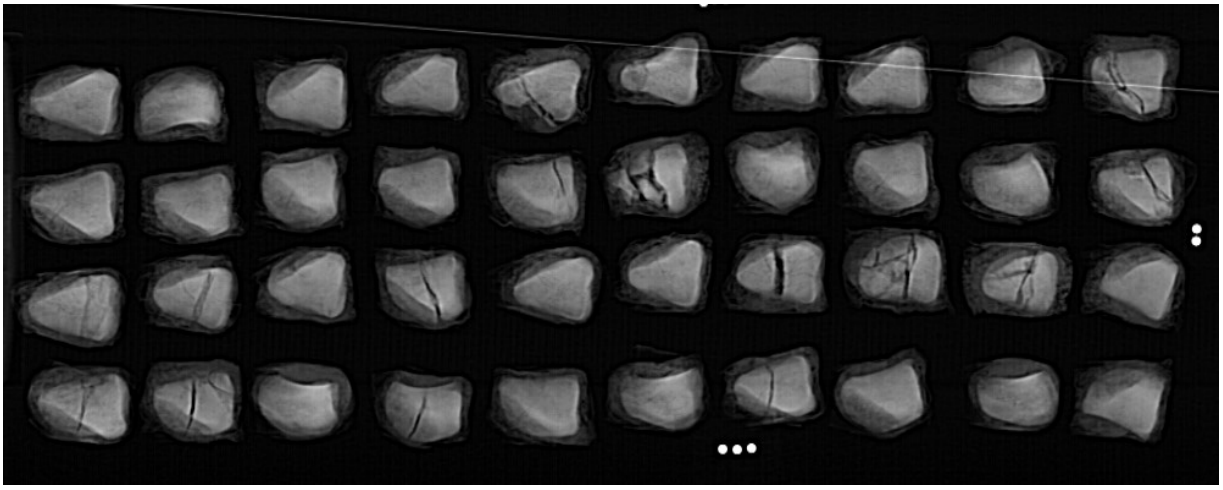

Figure 1. Scout CT scan of the group of PSBs from 10 horses euthanized as a result of fracture.

Radiographs (VTH) and MRI (3 Tesla – Purdue MRI facility; 7 Tesla – BME, Bindley Bioscience Center, Dr. Greg Tamer) were performed on forelimb PSBs in all 20 horses. Dissection and

removal of the soft tissues was performed prior to microCT analysis (140 um and 90 um resolution). Prior to destructive testing, the PSBs were imaged with a clinical CT 64 slice scanner GE with a 0.675 slice thickness and peripheral quantitative CT (pqCT) to correlate microCT findings with clinically available CT imaging modalities.

Proximal sesamoid bones are in processing for histology analysis of regional collagen fiber orientation (picosirius red), lacunocanalicular orientation (silver stain), osteoclast number (tartate-resistant acid phosphatase) and overall microstructure (H&E). Protocols, testing jig design and test samples for mechanical testing have been created. Currently, quantitative evaluations and statistical analysis of the µCT, 3T MRI, 7T MRI and pqCT imaging is being performed.

This project has been delayed for several reasons over the course of 2020, primarily through COVID related disruption in our activities (lab closures, remote work, disrupted clinical schedule with additional time commitments). In addition, Dr. Hermida had a short period of medical leave and has been working remotely as much as possible during this time due to his medical status. However, we also added to the project pqCT testing of the PSBs as an additional in vivo microstructural imaging method due to the availability of the modality to our group and its potential utility as an in vivo microstructural analysis technique for the horse (Desbrosse et al, Equine Vet Educ. 2008). This was not a part of the original description of work for this funding, but it has added a link to the other structural and mechanical information being collected for this project. The pqCT analysis is able to provide measures of material resistance to bending and moments of inertia in 3 different planes based on the microstructure, bone shape and material density. This unique aspect of pqCT may provide a link between our mechanical testing data and the other computed tomography imaging performed. We therefore elected to seize the opportunity to explore this imaging with this group of bones. Since the group of sesamoid bone fracture horses included in this study is a finite resource at this time, we elected to perform the pqCT testing prior beginning the destructive process of cutting and sectioning for histology and mechanical testing. The bones are now cut and in processing. Our final reporting on the complete data set is at least 6 months away at this time and we are happy to provide that update rather than consider this the final report if that is preferred. Publications should follow soon after this time as well as ultimately Dr. Hermida defending his work and thesis.

Respectfully submitted,

Timothy B. Lescun, BVSc, MS, PhD, Diplomate ACVS

Final Progress Report: Competitive Equine Research Funds (2018)

Mechanosensitive Channels in Equine Musculoskeletal Soft Tissues

PI: Dianne Little

Date of Award: 4/13/2018

Hypothesis: Transient receptor potential (TRP) channels and Piezo channel expression is upregulated in the palmar or plantar joint capsule of the fetlock joint with increasing severity of osteoarthritis, and in the flexor tendons and suspensory ligament with increasing evidence of suspensory desmitis or tendinopathy.

Specific Aim 1: Characterize the expression of mechanosensitive calcium TRP and Piezo channels across various sites in the equine fetlock joint and distal limb tendons from cadaveric donors of in different degrees of fetlock, tendon and ligament health.

Specific Aim 2: Characterize the functional role of mechanosensitive calcium channels in tendon and joint capsule extracellular matrix synthesis, matrix organization and contractility under simulated loading conditions.

Personnel:
Dr. Kara Negrini (PVM DVM'18), worked on this as her main project towards her Master's thesis requirement from May 2018 - May 2020. She graduated and moved to a residency program before COVID-19 restrictions were lifted sufficiently to allow her to complete the work.

Progress Towards Research Goals:
We made substantial progress on the research goals, to the point where the final set of experiments were underway, but unfortunately COVID-19 research ramp-down in March 2020 meant that 1) *in vitro* experiments had to be shut down, and 2) further tissue procurement was impossible. The cryostat was also damaged by an unauthorized user prior to COVID ramp down, which lead to several months of additional delays before COVID-19 restrictions were implemented. The CryoJane system previously evaluated to improve cryo-sectioning of equine tendons and joint capsule did not work adequately; instead we refined protocols using a different cryosection tape system (Kawamoto technique) that worked well.

All antibodies tested work well in equine positive control tissues, except for Piezo 2, and expression of TRPA1, TRPV1, TRPV4 and Piezo 1 was confirmed in normal dorsal and palmar fetlock joint from one horse with apparently differential expression of these

channels between capsule sites evaluated. Sectioning of remaining tissues was underway when COVID-19 ramp down was mandated. Continued studies with additional tissues will seek to validate these findings but could provide exciting opportunities for novel therapeutic modulation of joint capsule and potentially tendon under repetitive or injurious levels of mechanical loading.

Fibroblast isolation protocols from tendon and joint capsule were refined, and were successful, and in the limited number of collagen gel contraction assays possible before COVID-19 ramp down, the non-specific acid-sensing ion channel antagonist amiloride (5uM) stimulated contraction of collagen gels seeded with healthy dorsal joint capsule fibroblasts compared to negative and vehicle controls, an entirely unexpected finding. While these findings still need to be replicated, acid-sensing ion channels have an important role in inflammation and synovial hyperplasia in rheumatoid arthritis in humans, and tissue acidosis occurs in osteoarthritis, thus it is not unreasonable that acid-sensing ion channels could play a role in normal joint capsule homeostasis function and in maintenance of a non-fibrotic joint capsule state. Ongoing studies with fresh tendon and joint capsule fibroblast isolations will continue to explore this area.

Publications:
Dr. Negrini's Thesis was deposited with Purdue University and will be available after the end of the embargo period (May 2021).

Future Directions:
A new student will be sought once COVID-19 protocols are eliminated and once all laboratory personnel can be vaccinated, because quarantine or closure of the lab due to COVID exposure risks our existing valuable cell stocks being lost mid-experiment. The current data however, are adequate for development of an NIH funding application that will continue the work in equine as a model for human tendinopathy and joint capsule fibrosis. Thus these studies will ultimately significantly advance both human and equine sports medicine and orthopaedics.

ERAB Pilot Equine Research Funds, Progress Report, December 2021

Principal Investigator: Sandra D. Taylor

Co-Investigators: Dr. Janice Kritchevsky, Dr. Margaret Miller, Dr. Patrick Huang and Dr. Carla Olave

Title of Proposal: Understanding Midwest acute cholangiohepatitis

Project Summary

Beginning in November of 2020, veterinarians in the Midwest, including those at the Purdue University Veterinary Hospital (PUVH), identified horses with a previously-undescribed form of cholangiohepatitis. We have termed this Midwest Acute Cholangiohepatitis Syndrome (MACS). Clinical signs are limited to lethargy, anorexia, and markedly elevated rectal temperatures. Fevers persist for approximately five days, and the vast majority of horses experience a second cycle of high fevers after approximately one week of normothermia. Additional cycles are less common, but have been identified in some horses. Similar cases have been reported in Michigan, Illinois and Kentucky. Because there are stables with more than one animal affected, an infectious cause is believed most likely. However, despite extensive efforts, no pathogen has been identified. Various antibiotics have been given to affected horses although none appear to alter the clinical course; therefore, a viral etiology is suspected. Histopathology of liver biopsies consistently reveals cholangiohepatitis characterized by neutrophilic, lymphocytic and macrophagic inflammation as well as multifocal areas of necrosis. The goal of this study is to elucidate the clinical course and pathogenesis of disease following experimental transmission of blood from horses with MACS to healthy ponies. Given that viral hepatitis is associated with viremia in many species, including horses, transmission of blood during febrile episodes is expected to cause viremia and hepatitis in recipient horses.

Progress To-Date

No further cases were reported after funding of this project until December of 2021, at which time two additional acute cases were identified at the PUVH. However, the owners of these horses did not present the horses to the PUVH until after the febrile episodes; therefore, they were not eligible for blood donation. New cases will be recruited to complete the study and further investigate the etiology of this emerging, apparently seasonal disease.

Wilkes, R., Kattor, J. **Evaluation of Targeted Next Generation Sequencing for Detection of Equine Pathogens in Clinical Samples.**

- The investigators received a 1-year, no-cost extension for the project because of the impact of COVID-19 on their research activities.

APPENDIX C

Research Projects Completed Supported with Pari-Mutuel Funds:

- Figueiredo M, Lescun T, Gimble J. **Enhancing the Repair Potential of Equine-Derived MSC for Treating Post-Traumatic Osteoarthritis.**

- Hendrix, K., Kritchevsky, J**. Recovery of Salmonella Bacterial Isolates from Pooled Equine Fecal Samples.**

- Taylor, S. D., Anderson M, Reinhart J, Cooper B. **Pharmacokinetics of Thiamine Hydrochloride.**

Title: **Enhancing the repair potential of equine-derived MSC for treating post-traumatic osteoarthritis (PTOA)**

Investigators: **Marxa Figueiredo (PI, BMS), Tim Lescun (Co-I, VCS), Jeff Gimble (LaCell, Inc.)**

Date: **07-29-21**

Final Progress Report

The purpose of this project was to optimize the chondrogenic and anti-inflammatory potential of equine mesenchymal stem/stromal cells (eqMSC) by using a novel Laminin Receptor (LAMR1)-targeted small molecule, *compound C3*, for preventing post-traumatic osteoarthritis (PTOA) progression. Our preliminary data had showed promise for C3 in promoting chondrogenesis of equine ASC and BM-MSC and reducing inflammatory response in equine synoviocytes. However, the efficiency of C3 still must be optimized to the biology of equine MSC, in order to improve and maximize cartilage-specific and anti-inflammatory responses. We proposed to significantly optimize C3 efficacy and to test the hypothesis that equine MSC could be primed by compound C3 for enhanced chondrogenic and anti-inflammatory activity for preventing PTOA progression.

Aim 1. To examine whether the chondrogenesis and anti-inflammatory efficacy of compound C3 can be optimized for equine MSC in co-culture systems. Towards this Aim, we utilized equine synoviocytes. The MS student (Huff) developed new culture strategies and stimulation protocols to augment the inflammatory gene expression observed in eqSYN. She was able to stimulate synoviocytes towards an inflammatory gene expression response with LPS and equine TNFa, finding that TNFa was a much stronger stimulus. She also developed a real-time screen for testing the derivative compounds of C3, and preliminary data suggested that 02-09 derivative was better than the parental C3 compound at controlling expression of an IL1b-promoter-driven GFP reporter in this imaging-based screen. Her results are being included in her MS thesis, and future publication, when more compounds can be screened using this system.

Aim 2. To examine whether the chondrogenesis and anti-inflammatory efficacy of compound C3 can be optimized for equine MSC pre-treated with Poly I:C. We have explored the effect of PolyI:C in reducing inflammatory gene expression in ASC and worked on the experimental conditions to augment the effect on priming these cells with this TLR ligand. Our undergraduate student (Elsbury) assisted in moving this aim forward culturing ASC and BM-MSC from equine and stimulation with TLR3 agonist polyI:C. Her results were presented at the PVM Summer Scholars program this summer. The results will be incorporated in future publications on this project.

Other activities and immediate goals.

We have presented at least in 8 occasions, with several local posters and talks on this project, which is adequate considering COVID dramatically limited our travel capacity in the past 1.5 year

Posters/presentations: **A.** *VanSickle research day*: 1 talk by Dr Figueiredo, 1 poster (2nd place competition, by Danielle Keating, DVM 3rd year); **B.** *VCS ResearchTalks*, Dr. Figueiredo; **C.** *PVM research day*, 1 poster by Danielle Keating; **D.** *PULSe program Spring meeting*, 1 poster by Annika Robinson-Hudspeth). **E**, Krista Huff (BMS MS student), BMS692 seminar Fall 2019, Fall 2020; **F.** Krista Huff, Submitted abstract to present poster at Experimental Biology 2020 (San Diego, CA) for the

American Society of Biochemistry and Molecular Biology. G. Krista's thesis defense; H. Elsbury poster for the PVM Veterinary Scholars Summer Program.

Training and Mentoring. This grant afforded excellent opportunities for mentoring. We mentored three students in this grant (partially), Cosette Rivera, Krista Huff, and Claire Elsbury. The grad student (Cosette Rivera) won 1st place *Omicron Graduate Student Research Award* (PVM research day 2019). Krista Huff made significant contributions to Aim 1 and will soon be graduating with a MS from BMS (Fall 2021 expected). Claire Elsbury is an undergraduate in Biochemistry with an interest in a career as a DVM. She participated in the PVM Summer Research Program for undergraduates and presented her research on Aim 2.

Conferences. We would like to also disseminate the results soon in an orthopedic conference, and submit grants for pilot equine clinical trials following this project's completion to the Grayson Jockey Club Research and the American Quarter Horse Foundations. Current plans are to continue to prepare a manuscript for submission to a peer-reviewed journal in collaboration with the co-investigators (Lescun and Gimble) in 2021. There is also potential for leveraging the data into a new collaboration with Dr. Lescun and also Dr. Lynn Pezzanite of the Colorado State Veterinary Teaching Hospital as a collaborator, forming a new research team. We have recently met with her and are making plans to join forces for applying for a Grayson Jockey Club Research grant in the Fall of 2021 in the topic of this project.

Grant Applications. We applied for a total of 5 grants or related opportunities relating to this project, i.e. an Equine Fellowship for a PhD student (not successful), an AgSEED 2019 (successful), a PVM proposal (successful), USDA Hatch project (Successfully approved), and an AgSEED 2020 to extend translation to horses (not successful). We continue to utilize the equine cell and collaborative resources developed in this project, and will work with Dr. Lescun and Dr. Pezzanite as a collaborator to leverage our related projects to enhance our chances of obtaining further extramural funding.

Lay Article

Abstract. The relevance of this project to the Indiana horse industry is to develop drugs for treating or preventing post-traumatic osteoarthritis (PTOA) progression in equine athletes. These drugs already have some promising effects in cultures using cells isolated from equine athletes, such as mesenchymal stem cells from bone marrow and adipose tissue (fat) and synoviocytes. We propose to develop culture models that can mimic a 'joint in a dish' in order to better understand the therapeutic effect of our drugs on cartilage and in reducing inflammation. We also propose to examine whether we can enhance the ability of stem cells to repair joints using a molecule called Poly I:C, recently suggested as a way to 'license' stem cells towards enhanced joint repair. With these 'joint in a dish' and 'licensing to repair' approaches, we propose we can optimize stem cell therapy with our drugs for equine athletes suffering or at high risk for developing PTOA.

The long-term goals of the overall program. Within the past 2 years, our lab has become increasingly interested in MSC for anti-inflammatory and joint repair purposes, successfully pursuing a pilot project to bring our research approaches into equine MSC biology. Our goal is to incorporate our equine data into an extramurally funded application within the next year, in continued collaboration with Drs. Lescun and Gimble, and leveraging a new collaboration with Dr. Pezzanite of Colorado State. Target foundations may include the Grayson-Jockey Club Research, the American Quarter Horse, and/or the Morris Animal Foundations, which would facilitate translation of this work into the clinic.

ERAB Progress Report

"Recovery of Salmonella bacterial isolates from pooled equine fecal samples"

Dr. Kenitra Hendrix, Dr. Jose Goni and Dr. Janice Kritchevsky

Summary:

Current protocols for equine Salmonella culture include testing a series of five samples, usually collected at 24-hour intervals. The purpose of this study is to evaluate the sensitivity of culturing pools of five fecal samples for Salmonella culture. Testing pooled samples would offer the benefit of decreased cost of diagnostic testing.

Complete:

1. *Obtain and propagate a pure culture of Salmonella Group E to serve as the reference bacteria.* - complete

Salmonella E1 (ATCC 9270) is maintained in the ADDL Bacteriology section and utilized in subsequent specific aims.

2. *Spike equine fecal samples with either 10^2, 10^3, 10^4, or 10^5 CFU Salmonella.*
3. *Pool 1 spiked and 4 non-spiked fecal samples collected over a 5-day time period into a single container.*
4. *Perform standard culture to recover the spiked Salmonella species from well-mixed composite fecal samples.*

Two iterations of aims 2-4 were performed.

Phase 1 (Table 1): A series of five Salmonella samples from the feces donor horse were cultured and were negative for Salmonella. Feces in 20-gram aliquots from this Salmonella negative horse was initially spiked with either 10^2, 10^3, 10^4, or 10^5 CFU Salmonella. Ten grams were cultured for salmonella to show spiking was successful. The other ten grams were pooled with 40g of salmonella-negative feces, replicating a pool of one positive field samples and four negative field samples. Following both spiking and pooling, feces was homogenized for 1 minute at 230rpm. All spiking was successful based on positive culture results. All pools were positive, with the exception of the pool including the samples spiked with 10^3 CFU. This raised concerns regarding the pooling technique itself, since the pool with less organism was culture positive. All pooled samples were cultured again after 8 days at 4°C, to simulate a field case in which only the first sample was positive for Salmonella. All pools were positive except for the one including the sample spiked with 10^2 CFU.

Table 1:

		First Culture	Second Culture (8d in fridge)
A	20g feces spiked with **10^2** CFU	Salmonella	----------
AP	10g A pooled with 40g negative	Salmonella	Negative
B	20g feces spiked with **10^3** CFU	Salmonella	----------
BP	10g B pooled with 40g negative	Negative	Salmonella
C	20g feces spiked with **10^4** CFU	Salmonella	----------
CP	10g C pooled with 40g negative	Salmonella	Salmonella
D	20g feces spiked with **10^5** CFU	Salmonella	----------
DP	10g D pooled with 40g negative	Salmonella	Salmonella (Group E-1)

Phase 2 (Table 2): In response to the unexpected results in phase 1, variations of homogenization protocols including the duration of homogenization and adding nutrient broth were tested. Samples were spiked with 10^2 CFU only, and then pooled as described in Phase 1. Triplicates of each set of conditions were tested, and all culture results were positive. This indicated that variables of the homogenization protocol did not affect the final culture results, and that 10^2 CFU in 50g of feces was consistently detected by culture.

Table 2:

Control	Non-spiked feces	Negative
1a	1 minute; no broth	Positive
1b		Positive
1c		Positive
5a	5 minutes; no broth	Positive
5b		Positive
5c		Positive
B1a	1 minute; 20mL broth	Positive
B1b		Positive
B1c		Positive
B5a	5 minutes; 20mL broth	Positive
B5b		Positive
B5c		Positive

In-progress:

A. Additional spiking study to determine the best pooling protocol:
   - 12 10-gram samples will be spiked with 10^2 CFU/mL *Salmonella* (ATCC 9270)
   - Each spiked sample will be pooled with 40g of *Salmonella*-negative samples, and homogenized according to Table 2 above.
   - Each 50g spiked pool will be divided into 5 10-gram samples, and each will be cultured for *Salmonella* according to ADDL protocol.

B. For one calendar year, each time a series of 5 equine fecal samples is cultured for *Salmonella*, duplicate samples will be saved in the VTH and submitted as a single pooled sample for culture.
   - The fecal pool will be homogenized per the protocol selected in A.
   - 10g of the pool will be cultured for Salmonella according to the ADDL protocol.

CVM Pilot Equine Research Funds (2019), Final Report

March, 2021

Principal Investigator: Sandra D. Taylor

Co-Investigators: Dr. Melinda Anderson, Dr. Jennifer Reinhart and Dr. Bruce Cooper

Title of Proposal: Pharmacokinetics of thiamine hydrochloride (Vitamin B1) in adult horses

Project Summary

Sepsis is a major cause of morbidity and mortality in neonatal foals and adult horses. Effective treatment of sepsis requires that the excessive inflammatory response, known as Systemic Inflammatory Response Syndrome (SIRS), is interrupted. Thiamine is a vital co-factor in many metabolic processes, including those that are critical in mitigating inflammation. Previous studies in humans have shown that metabolic resuscitation, a term used to reference the use of thiamine hydrochloride (TH), ascorbic acid, and hydrocortisone, decreases inflammation and increases survival of septic patients. The benefits of TH in treating diseases in horses are unknown. Before efficacy studies of TH alone or TH in combination with ascorbic acid and hydrocortisone can be performed in horses, pharmacokinetic (PK) analysis is necessary. We hypothesized that intravenous (IV) TH at increasing doses results in corresponding increases in plasma TH concentrations without causing adverse effects.

Progress to Date

A PK analysis of 3 doses of TH was performed. A randomized cross-over study included 9 healthy adult horses treated with IV TH at 5, 10, and 20 mg/kg. For each treatment, blood was collected immediately prior to drug administration (T0) and 5, 10, 15, 20, 30, 45, 60, 90 minutes and 2, 4, 6, 8, 10, 12, 24, and 48 hours after drug administration. A physical examination was completed at T0, 6, 12, 24 and 48 hours. No clinical signs of adverse effects were observed.

Intravenous TH administration resulted in supraphysiologic plasma concentrations with a relatively short half-life (0.77 – 1.12 h) and no adverse effects were observed. A time vs. concentration curve is shown in **Figure 1**. When comparing the 20 mg/kg and 5 mg/kg dosages, Cl significantly decreased at the 20 mg/kg dosage ($p=0.0011$). However, the Cl at the 10 mg/kg dosage was significantly higher than the 5 mg/kg ($p=0.0388$) and 20 mg/kg ($p=0.0004$) dosages. The findings suggest TH follows non-linear elimination kinetics in horses and is likely due to saturation of elimination processes, likely renal excretion. Future pharmacodynamic studies are needed to identify therapeutic plasma concentrations in order to establish rational dosing regimens.

A manuscript has been started and submission for publication is expected by June of 2021.

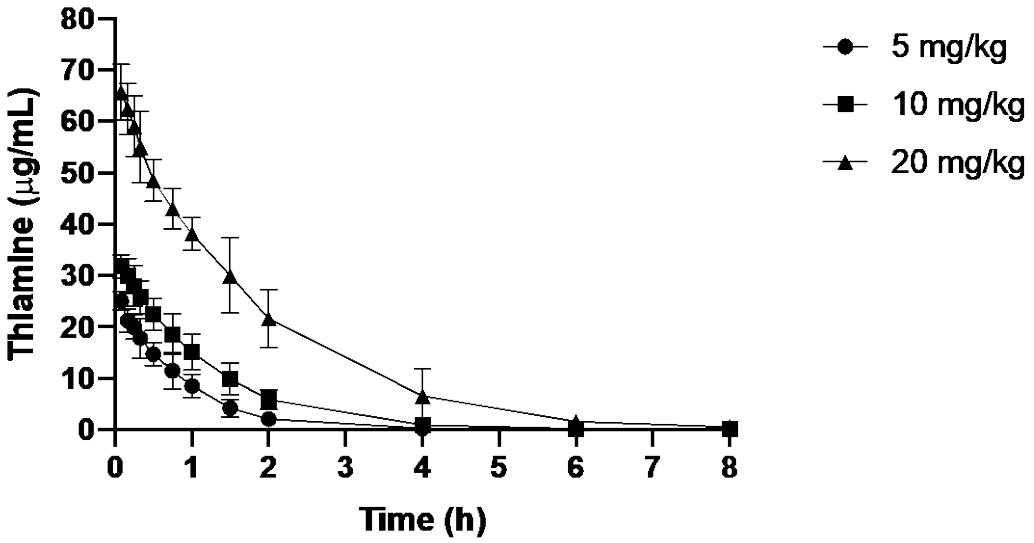

Figure 1. Thiamine concentration vs. time after IV administration of thiamine hydrochloride at 5, 10 or 20 mg/kg to healthy horses (n=9).

CVM Pilot Equine Research Funds (2019), Final Lay Report

March, 2021

Principal Investigator: Sandra D. Taylor

Co-Investigators: Dr. Melinda Anderson, Dr. Jennifer Reinhart and Dr. Bruce Cooper

Title of Proposal: Pharmacokinetics of thiamine hydrochloride (Vitamin B1) in adult horses

Project Summary

Sepsis is a major cause of illness and death in horses. Effective treatment of sepsis requires that the excessive inflammatory response, known as Systemic Inflammatory Response Syndrome (SIRS), is interrupted. Thiamine is a vital co-factor in many metabolic processes, including those that are critical in decreasing inflammation. Previous studies in humans have shown that a combination of thiamine hydrochloride (TH), ascorbic acid (Vitamin C), and hydrocortisone (steroid) decreases inflammation and increases survival in septic patients. The benefits of TH in treating diseases in horses are unknown. Before efficacy studies of TH alone or TH in combination with ascorbic acid and hydrocortisone can be performed in horses, pharmacokinetic (PK) analysis is necessary to determine how the drug is metabolized in the body and how quickly this occurs.

Progress

A PK analysis of 3 different doses of TH was performed. These doses were extrapolated from TH studies in other veterinary species, as well as dosing recommendations for septic people. Nine horses were given IV TH at each of 3 doses (5, 10, and 20 mg/kg), with 1 week separating each dose to allow drug to be cleared from the system. For each treatment, blood was collected immediately prior to drug administration (T0; baseline) and 5, 10, 15, 20, 30, 45, 60, 90 minutes and 2, 4, 6, 8, 10, 12, 24, and 48 hours after drug administration. These samples were used to test thiamine concentration in the blood. A physical examination was completed at T0, 6, 12, 24 and 48 hours. No side effects were observed.

Intravenous TH did not stay in the blood stream for long (half-life = approximately 1 hour). The middle dosage (10 mg/kg) resulted in the highest plasma concentrations and there was evidence that the body's ability to eliminate the drug "maxes out." Future studies are needed to determine the therapeutic dose of thiamine for horses with sepsis.

A manuscript has been started and submission for publication is expected by June of 2021.

APPENDIX D

Refereed Scientific Articles:

- Couetil, L.L., Ivester, K., Barnum, S., Pusterla, N. **Equine Respiratory Viruses, Airway Inflammation and Performance in Thoroughbred Racehorses.** *Vet Microbiol*. 2021 Jun;257:109070. doi: 10.1016/j.vetmic.2021.109070. Epub 2021 Apr 10.

- Gray, S., Gutierez-Nibeyro, S.D., Couëtil, L.L., Secor, E.J., Schaeffer, D.J. **Evaluation of the Airway Mechanics of Modified Toggle Laryngoplasty Constructs using a Vacuum Chamber Airflow Model.** *Vet Surg*. 2021 Jul 26. doi: 10.1111/vsu.13690. Epub ahead of print.

- Hess, E.K., Reinhart, J.M., Anderson, M.J., Jannasch, A.S., Taylor, S.D. **Pharmacokinetics of Thiamine (Vitamin B1) in Adult Horses after Administration of Three Single Intravenous Doses.** *J Vet Pharmacol Ther.* 2021 Nov;44(6):937-944. doi: 10.1111/jvp.13007. Epub 2021 Aug 18.

- Kritchevsky, J.E., Olave, C., Tinkler, S.H., Tropf, M., Ivester, K., Forsythe, L., Couëtil, L.L. **A Randomised, Controlled Trial to Determine the Effect of Levothyroxine on Standardbred Racehorses.** *Equine Vet J*. 2021 Jun 8. doi: 10.1111/evj.13480. Epub ahead of print.

- Olave, C.J., Ivester, K.M., Couëtil, L.L., Kritchevsky, J.E., Tinkler, S., Mukhopadhyay, A. **Dust Exposure and Pulmonary Inflammation in Standardbred Racehorses Fed Dry Hay or Haylage: A Pilot Study.** *Vet J.* 2021. May;271:105654. https://doi.org/10.1016/j.tvjl.2021.105654.

- Olave, C.J., Ivester, K.M., Couëtil, L.L., Franco, J., Mukhopadhyay, A., Robinson, JP., Park, J.H. **Effects of Forages, Dust Exposure and Pro-Resolving Lipids on Airway Inflammation in Horses.** Am J Vet Res. Doi: 10.2460/ajvr.21.08.0126. Epub 2021 Nov 25.

- Serpa, P.B.S., Woolcock, A., Taylor, S.D., Pires Dos Santos, A. **Validation of a Flow Cytometric Assay to Detect Intraerythrocytic Reactive Oxygen Species in Horses.** *Vet Clin Pathol*. 2021 Mar;50(1):20-27. doi: 10.1111/vcp.12976. Epub 2021 Mar 1.

- Taylor, S.D., Ivester, K.M., Stewart, C., Horohov, D., Couëtil, L.L. **The Effect of Lower Airway Inflammation on Inflammatory Cytokine Gene Expression in Bronchoalveolar Lavage Fluid and Whole Blood in Racing Thoroughbreds.** *Vet Immunol Immunopathol*. 2021 Jul;237:110266. doi: 10.1016/j.vetimm.2021.110266. Epub 2021 May 9.

- Taylor, S.D., Kritchevsky, J.E., Slovis, N.M., Wilkins, P.A., Austin, S.M,. Schott, H.C. **Emerging Outbreak of Hepatitis in Midwestern Horses.** Letter to Editor, *J Am Vet Med Assoc*. 2021;258(11):1183.

- Taylor, S.D., Serpa, PBS, Santos, A.P., Hart, K.A,. Vaughn, S.A., Moore, G.E., Mukhopadhyay, A., Page, A.E. **Effects of Intravenous Administration of Peripheral Blood-Derived Mesenchymal Stromal Cells after Infusion of Lipopolysaccharide in Horses.** *J Vet Intern Med*. Under Final Review, December 2021.

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Equine respiratory viruses, airway inflammation and performance in thoroughbred racehorses

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ABSTRACT

Equine asthma is a common cause of poor performance in racehorses but it is unclear if respiratory viruses contribute to its etiology. The objective of the study was to determine if respiratory viruses were associated with clinical signs and bronchoalveolar lavage fluid (BALF) cytology in Thoroughbred racehorses. Equine herpesviruses (EHV-1, 2, 4, 5) and equine rhinitis A and B viruses (ERBV, ERAV) genomes were quantified by qPCR in nasopharyngeal, tracheal, and BALF samples collected after racing. The relationships between virus detection and load and clinical signs, performance, BALF cytology, and environmental exposures were examined with generalized linear mixed models. Ninety-two samples were collected from 31 horses. EHV-1 and ERAV were not found; EHV-4 was detected in only one sample. EHV-2, EHV-5 and ERBV were more likely to be detected in upper airway samples than in BALF ($P < 0.0001$). Neither respiratory virus detection nor load was associated with clinical signs or performance. Nasopharyngeal detection and load of ERBV and tracheal detection and load of EHV-5 were associated with increased proportions of neutrophils in BALF ($P < 0.003$). However, nasopharyngeal detection and load of EHV-5 was not ($P = 0.11$). Nasopharyngeal detection and load of EHV-2 were associated with decreased BALF mast cell proportions. Respirable dust exposures were significantly higher in horses with detection of ERBV when compared to horses with no detectable ERBV ($P < 0.001$). Our results suggest that ERBV, EHV-2 and EHV-5 are commonly present in upper airways of healthy racehorses; however, the role they play in the etiology of equine asthma remains unclear.

1. Introduction

Mild equine asthma, previously referred to as inflammatory airway disease (IAD), may affect up to 80–90 % of racehorses based on bronchoalveolar lavage fluid (BALF) cytology (Depecker et al., 2014; Ivester et al., 2018). Clinical signs are usually subtle including decreased performance and mild intermittent coughing in the absence of fever and other systemic signs of illness (Couëtil et al., 2016). Diagnosis is made by ruling out other causes of poor performance or coughing and documenting lower airway inflammation by endoscopic scoring of tracheal mucous or cytology of BALF, where proportions of neutrophils, mast cells, and/or eosinophils are increased (Couëtil et al., 2016).

The etiology of mild equine asthma remains incompletely understood and both infectious and non-infectious agents have been implicated. Some studies have documented an association with exposure to small dust particles and neutrophilic airway inflammation (Millerick-May et al., 2013; Ivester et al., 2018). Organic components within the dust appear to also play a role. Specifically, β-glucan, a component of fungal and plant cell walls, can be measured as an indicator of fungal exposure and has been associated with increased BALF proportions of mast cells (Ivester et al., 2018). Other studies have identified a link with bacterial colonization of the trachea (Christley et al., 2001; Wood et al., 2005; Cardwell et al., 2014) or viral respiratory infections (Fortier et al., 2009a; Doubli-Bounoua et al., 2016). Acute respiratory infections with equine influenza virus and equine herpes viruses (EHV-1 and EHV-4) are common cause of fever and systemic illness in racehorses (Gilkerson et al., 2015). Horses with mild equine asthma do not show signs of systemic disease; nevertheless, the potential role of respiratory viruses has been investigated using serological testing or detection of viral genome in airway secretions by PCR. So far, the evidence is controversial, with some studies showing a link between mild equine asthma and respiratory infection with EHV-2, EHV-5, equine rhinitis B

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0378-1135/

virus (ERBV) (Fortier et al., 2009a; Doubli-Bounoua et al., 2016) while others do not (Newton et al., 2003; Back et al., 2015a).

To date, virus prevalence in respiratory secretions of mild asthmatic horses racing in the United States has not been reported. Therefore, the goals of this study were to determine: 1) the presence of virus genome and load in nasal, tracheal and bronchoalveolar samples collected from Thoroughbreds 1 h after racing; 2) the relationship between viral detection and load between different respiratory sites; 3) the association between viral detection or load and airway inflammation based on BALF cytology.

2. Materials and methods

2.1. Study design

This study was nested within a larger prospective observational study that was previously reported (Ivester et al., 2018). Trainers were contacted and permission for the enrollment of each Thoroughbred horse was obtained at least 24 h prior to racing. An informed consent form was signed prior to the race. Approximately 1 h after the horse completed the race, physical examination, endoscopy of the respiratory tract, nasopharyngeal and tracheal brushing, and BAL were performed. Horses were eligible for re-enrollment with every race entered. Performance was assessed as the speed figure (Equibase[1]) assigned to the horse after the race and is based on the horse's speed adjusted for distance and various racetrack factors (Ivester et al., 2018). The Purdue University Animal Care and Use Committee (protocol # 1,111,000,181) and the Indiana Horse Racing Commission approved all procedures.

2.2. Airway endoscopy and brushing

Horses were sedated with with butorphanol (0.02–0.04 mg/kg IV; [Torbugesic][2]) and with xylazine hydrochloride (0.2–0.5 mg/kg IV; [AnaSed][3]). A 1.5 m-long, 7.9 mm OD flexible video endoscope was passed through the ventral meatus to the level of the nasopharynx and the degree of pharyngeal lymphoid hyperplasia was scored from 0 (no follicles) to 4 (numerous, large, edematous follicles) (Auer et al., 1985). A sterile, guarded cytology brush[4] ([Disposable Cytology Brush], OD: 2.5 mm; length: 180 cm) was introduced through the working channel of the endoscope and deployed once in the nasopharynx. The brush was exteriorized from the protective sheath and gently rubbed back and forth 5 times against the mucosa at each of four locations (soft palate, left, right and dorsal walls of the nasopharynx). Then, the brush was retracted into its protective sheath and retrieved from the endoscope. The endoscope was subsequently advanced to mid-trachea and mucus and exercise-induced pulmonary hemorrhage (EIPH) scored according to Gerber et al. (Gerber et al., 2004) and Hinchcliff et al. (Hinchcliff et al., 2005), respectively. A new cytology brush was inserted through the working channel of the endoscope and mucosa sampled as described above (dorsal, ventral, right, and left tracheal wall). After sample collection, each cytology brush was exteriorized from the protective sheath and agitated for 1 min in a sterile vial containing 1 mL of transport solution ([RNAlater][5]). The vial was kept at −80 °C until transport to the laboratory for extraction and viral analysis.

2.3. Bronchoalveolar lavage

A sterile BALF tube[6] (300 cm long; 10 mm outer diameter) was used to perform the BAL (Ivester et al., 2018). Two hundred and fifty (250) mL of sterile 0.9 % sodium chloride were instilled and recovered using 60 mL syringes. Two mL of the final aliquot recovered were aseptically transferred to a dry sterile vial and frozen at −80 °C until transport to laboratory for extraction and viral analysis. Remaining BALF was pooled and placed on ice. Cytospin slides were prepared within 4 h and processed with modified Wright stain. Differential cell counts were determined by enumerating 600 cells per horse.

2.4. Environmental exposure assessment

Gravimetric filter sampling was conducted 4–7 days after racing as previously described (Ivester et al., 2018). Briefly, the respirable fraction (50 % cutoff of 4 μm) was collected from the breathing zone using an aluminum cyclone (SKC, Inc, Eighty Four, PA) and personal sampling pumps (Aircheck 2000, SKC, Inc, Eighty Four, PA) onto preconditioned 37 mm type AE glass fiber filters. Respirable dust measurements were determined gravimetrically by subtracting the average of three weights taken before sampling from the average of three weights taken after sampling. Filters were conditioned in a dessicator for 24 h before weight measurements were obtained. Filters were stored at −20 °C until elution for β-glucan analysis.

β-glucan content of respirable dust was measured using a kinetic chromogenic limulus amebocyte lysate technique (NexGen PTS, Charles River Laboratories Wilminton, Mississipi) as previously described (Ivester et al., 2018).

2.5. Nucleic acid extraction and PCR assays

All viruses were detected at the RNA level following the synthesis of cDNA. All viruses were tested at the complementary (c)DNA level. Extraction of viral nucleic acids was performed using the QIAamp® Viral RNA Kit[7] according to the manufacturer's instructions. Complementary DNA from each sample was synthesized using 50 U SuperScript III (Invitrogen, Carlsbad, CA) in a 40 mL final volume containing 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 0.5 mM dNTPs, 40 U RNAsin, 0.5 mM dithiothreitol (DTT) and 600 ng random hexadeoxyribonucleotide (pd(N)6) primers (random hexamers). The reaction was performed at 50 °C for 60 min. After inactivation at 95 °C for 5 min, the reaction volume was adjusted to 100 mL with nuclease-free water. Samples were stored at −80 °C until assayed. Virus specific qPCR were performed using previously reported assays (Bell et al., 2006; Mori et al., 2009). Positive and negative controls were used for every qPCR assay, and nucleic acid quality was assessed by analyzing all extracted biological samples for the presence of the housekeeping gene equine glyceraldehyde-3-phosphate dehydrogenase (eGAPDH). Absolute quantitation of EHV-1, 2, 4, 5, ERAV and ERBV target molecules was performed using standard curves and eGAPDH and expressed as number of target gene for the respective virus per million cells (Pusterla et al., 2009).

2.6. Data analysis

Generalized linear mixed models were constructed to determine the likelihood of detecting virus or virus load (log-transformed data) based on sampling site, trainer, month of sampling, and horse's age and gender. Models were also constructed to examine the effect of virus detection and virus load upon performance (Ivester et al., 2018), pharyngeal hyperplasia score, EIPH score and tracheal mucus score, and BALF cytology (neutrophil proportions and mast cells proportions), controlling

[1] Equibase, Lexington, Kentucky, USA.
[2] Zoetis, Parsippany-Troy Hills, New Jersey, USA.
[3] Akorn Animal Health, Lake Forest, Illinois, USA.
[4] Endoscopy Support Services, Inc., Brewster, New York, USA.
[5] RNAlater, Sigma-Aldrich, St. Louis, Missouri, USA.
[6] Bivona, Gary, Indiana, USA.
[7] Qiagen, Inc., Santa Clara, CA.

for age and trainer. Finally, environmental exposures (respirable dust and respirable β-glucan concentrations) were compared between horses with and without detection of viral genomes. Finally, exposure variables were then included in those models demonstrating association between BALF cytology and viral load in order to investigate any confounding effects between environmental exposures and viral load.

3. Results

Nasopharyngeal and tracheal brushings and BALF samples were obtained from 31 horses (92 samples; 1 nasopharyngeal brush missing). Horses were under the care of five different trainers and were housed in five separate barns.

3.1. Virus detection and sampling site

None of the samples were positive for EHV-1 or ERAV and only one sample was positive for EHV-4 (nasopharyngeal brush). There was a higher likelihood of detecting EHV-2, EHV-5 and ERBV in nasopharyngeal samples compared to other sites (Table 1; $P < 0.0001$) but no association with horse's age or sex. EHV-5 was also more likely to be detected in tracheal samples than BALF ($P < 0.0001$). EHV-2 was less likely to be detected in samples collected from trainer 1 horses ($P = 0.046$).

Viral load ranges were wide for EHV-2 (1237 – 1,666,171 gene copies per million cells) and EHV-5 (480–641,922 gene copies per million cells). Viral load for EHV-2 and EHV-5 were not significantly different between sampling sites (Fig. 1). Log transformed EHV-5 load did not vary by barn, trainer or horse's age, but did vary by month of the year (Fig. 2; $P = 0.014$). EHV-5 load was lowest in respiratory samples collected during the month of May and increased each subsequent month until it reached a plateau in September/October (end of the race meet first week of November).

Three viruses (EHV-2, EHV-5, ERBV) were identified together in nasopharyngeal samples from 19 horses, tracheal samples from 3 horses and BALF from only 1 horse. One horse had the three viruses detected

Table 1
Percentage of respiratory samples where viruses were detected by qPCR. EHV: Equine herpesvirus. ERAV: Equine rhinitis A virus. ERBV: Equine rhinitis B virus. *: increased likelihood of virus detection in nasopharynx compared to other sites ($P < 0.001$).

Viruses	Nasopharynx	Trachea	Bronchoalveolar lavage
EHV-1	0	0	0
EHV-2	77.4% (24/31)*	29.0 % (9/31)	12.9 % (4/31)
EHV-4	3.2 % (1/31)	0	0
EHV-5	83.4% (26/31)*	50.0 %(15/30)	16.7 % (5/30)
ERAV	0	0	0
ERBV	64.5 % (20/31)*	6.7 % (2/30)	3.2 % (1/31)

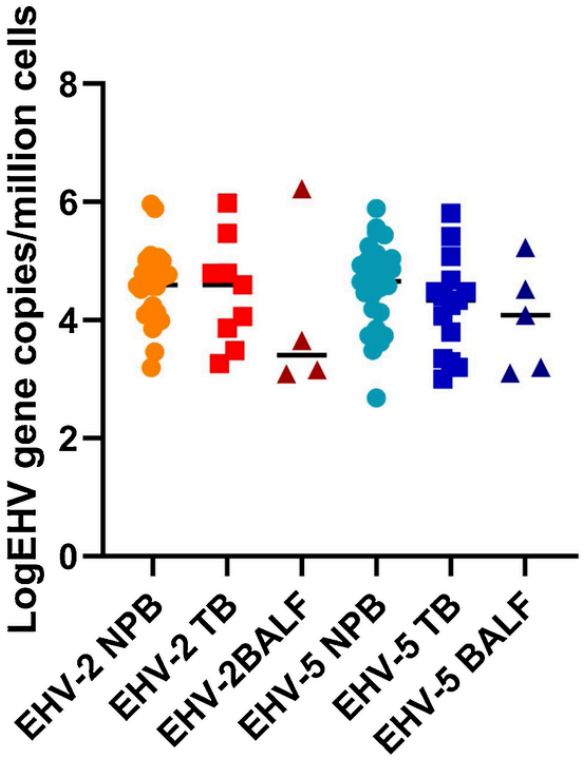

Fig. 1. Viral load for equine herpesvirus-2 (EHV-2) and EHV-5 in respiratory secretions collected by nasopharyngeal brushing (NPB), tracheal brushing (TB) and bronchoalveolar lavage (BAL). Data displayed as log-transformed EHV gB gene copies per million nucleated cells.

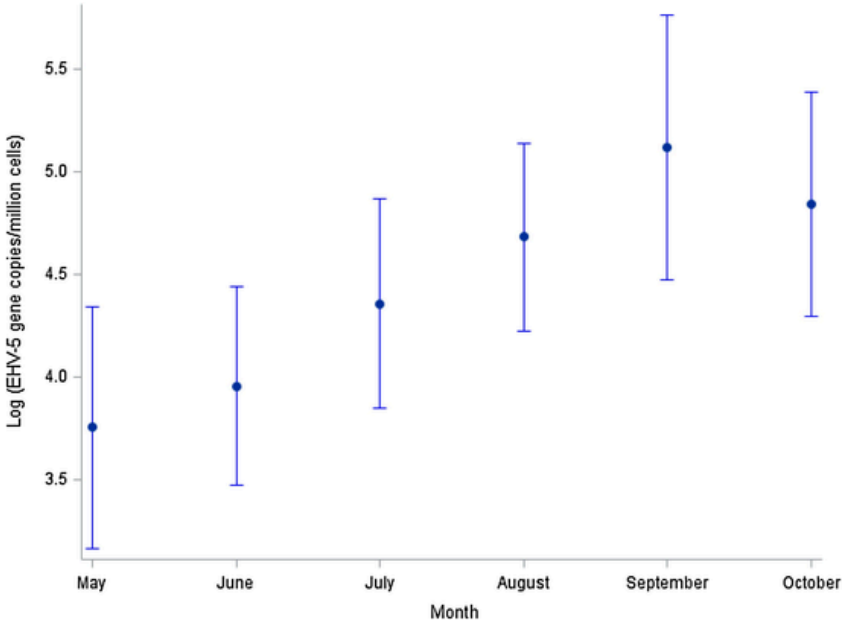

Fig. 2. Relationship between log transformed EHV-5 genome load and month of the year from May to October ($P = 0.014$).

from samples collected at all levels of the respiratory tract. This horse was a 3-year old Thoroughbred who finished the race in fourth place and had physical examination and BALF cytology within the reference range. Both EHV-2 and EHV-5 were identified in nasopharyngeal samples from 23 horses, tracheal samples from 6 horses and BALF from 2 horses.

3.2. Virus detection and clinical signs

Horse's performance as measured by the speed figure and adjusted for age was not associated with detection of viruses or virus loads in any of the respiratory samples ($p > 0.1$). Similarly, there was no association between EIPH ($p > 0.2$) or pharyngeal lymphoid hyperplasia scores ($p > 0.09$) and detection of viruses or virus loads in respiratory samples, controlling for horse's age. EHV-5 virus load in BALF was associated with a lower mucus score ($P = 0.024$), but neither presence of virus nor virus load in any other sample were associated with mucus score ($p > 0.06$).

3.3. Virus detection and BALF cytology

Detection of ERBV ($P = 0.0013$) and ERBV load ($P = 0.0024$) in nasopharyngeal brushings were associated with increased neutrophil proportions in BALF. Inclusion of respirable dust exposure as an explanatory variable had a profound effect upon the later model: when controlling for respirable dust exposure, ERBV load in nasopharyngeal brushings, was negatively associated with neutrophil proportions and no longer statistically significant ($p = 0.74$). As only 2 tracheal samples and 1 BALF sample were positive for ERBV, the effect upon BAL cytology was not modeled. Detection of EHV-5 ($P = 0.0026$) and virus load ($P = 0.0013$) in trachea were associated with increased BALF neutrophil proportions, while detection of EHV-5 in the nasopharynx was not ($p = 0.11$). In contrast, detection of EHV-5 ($P = 0.0061$) and virus load ($P = 0.0064$) in BALF were negatively associated with BALF neutrophil proportions. Inclusion of respirable dust exposure as an explanatory variable in these models had minimal effect on model interpretation: when controlling for respirable dust exposure, EHV-5 load in tracheal brushings remained positively associated with neutrophil proportions ($p = 0.025$) while EHV-5 load in BAL samples remained negatively associated with neutrophil proportions ($p = 0.0223$) Detection of EHV-2 or virus load in any of the sampling sites was not linked to BALF neutrophil proportions.

Detection of EHV-2 ($P = 0.018$) and EHV-2 load ($P = 0.021$) in nasopharyngeal brushings were associated with lower BALF mast cell proportions and this relationship was influenced by the horse's age ($P = 0.04$). When controlling for β-glucan exposure, EHV-2 load in nasopharyngeal brushings remained negatively associated with BALF mast cell proportions, but was no longer statistically significant ($p = 0.33$), with measures of β-glucan exposure unavailable for 10 horses. Detection of ERBV or EHV-5 or virus loads at any sampling site were not linked to mast cell proportions in BALF.

3.4. Virus detection and environmental exposures

Only detection of ERBV was associated with significantly higher respirable dust exposure ($P < 0.001$). Respirable β-glucan exposures did not differ significantly between horses with and without detection of ERBV, EHV-2, or EHV-5 ($P > 0.2$, Table 2).

Table 2
Respirable dust and respirable β-glucan mean (s.e.m.) exposures as measured at the breathing zone.

			Respirable Dust (mg/m^3)	Respirable β-glucan (pg/m^3)
ERBV2	Not Detected	n = 8	0.00884 (0.00361)*	36.7 (18.94)
	Detected	n = 16	0.106 (0.0422)*	19.4 (6.86)
EHV-2	Not Detected	n = 5	0.0315(0.0175)	50.5 (35.9)
	Detected	n = 19	0.0471 (0.0145)	20.1 (6.39)
EHV-5	Not Detected	n = 5	0.0519 (0.0284)	13.8 (9.74)
	Detected	n = 19	0.0410 (0.0126)	27.3 (8.61)

* $p < 0.0001$.

4. Discussion

The present report is the first prospective study investigating detection of respiratory viruses simultaneously in nasal, tracheal and bronchoalveolar samples of horses. The main finding of this study was that EHV-2, EHV-5 and ERBV were commonly identified by qPCR in upper airway samples collected from healthy Thoroughbred racehorses one hour after racing but were rarely detected in BALF. Results are consistent with studies that have reported frequent detection of EHV-2 and EHV-5 genome in nasal or nasopharyngeal swabs (EHV-2: 30–76 %; EHV-5: 74–91 %; ERBV: 1–8 %) and tracheal wash (EHV-2: 11–35 %; EHV-5: 0–55 %; ERBV: 8 %) samples collected from racehorses in Europe (Fortier et al., 2009a, b; Back et al., 2015a, b; Doubli-Bounoua et al., 2016). These viruses are also identified in nasal or tracheal secretions from healthy sport horses (EHV-2: 0–18 %; EHV-5: 0–41 %) (Fortier et al., 2009a; Pusterla et al., 2013; Houtsma et al., 2015). The ERBV genome was detected in the nasopharynx of healthy Thoroughbred racehorses at a higher frequency (65 %) than in other studies of healthy racehorses or sport horses (0–8 %) (Pusterla et al., 2013; Houtsma et al., 2015; Back et al., 2015a; Doubli-Bounoua et al., 2016). The current study was unique because nasopharyngeal samples were collected using a protected brush inserted through the working channel of the endoscope whereas other studies have used swabs to collect either nasal or nasopharyngeal samples (Pusterla et al., 2013; Houtsma et al., 2015; Back et al., 2015a; Doubli-Bounoua et al., 2016). Studies comparing various methods of collecting respiratory samples for detection of viral genome in humans report either similar yield between nasal swab and nasal brush (Spyridaki et al., 2009) or better performance of nasal swab (Hou et al., 2020). Other potential explanations are that Thoroughbred racehorses are more susceptible to ERBV infection because previous studies were performed in Standardbred racehorses (Back et al., 2015a; Doubli-Bounoua et al., 2016) or that ERBV infection rate is higher in horses racing in the US than in Europe despite an apparently low infection rate in sports horses in the US (Pusterla et al., 2013; Houtsma et al., 2015). Detection of ERBV in tracheal samples (7 %) was similar to previously reported in healthy trotters in Europe (Doubli-Bounoua et al., 2016).

Although EHV-2, EHV-5 and ERBV were frequently detected in upper airway samples, we were not able to find an association between virus presence or load and performance. Horse's performance is strongly influenced by age (Barrey, 2010; Ivester et al., 2018); therefore, adjusting data analysis for the horse's age, as done in the present study, is important. Our findings are consistent with a study involving 66 trotting racehorses followed monthly over 13 months that reported no association between EHV-2, EHV-5 and ERBV detection or load in nasal secretions and performance as assessed by trainers' opinion or standardized exercise test (Back et al., 2015a, b). In addition, we did not find any association between clinical signs of respiratory disease and virus detection or load in the nasopharynx or trachea of Thoroughbred racehorses. The only exception was the association between EVH-5 virus load in BALF and lower mucus score. Only 5 out of 30 BALF samples had detectable EHV-5 therefore, we need to use caution when interpreting these findings. A link between detection of EHV-2, EHV-5 and ERBV in airway samples and clinical signs of respiratory disease is controversial. Some studies found no relationship (Brault et al., 2011; Back et al., 2015a, b) but others identified an association between

coughing and detection of EHV-2 and ERBV genome in tracheal secretions or between EHV-2 and excess tracheal mucus (Doubli-Bounoua et al., 2016). Studies reporting association between respiratory viruses and clinical signs do not establish causality as results may be influenced by confounding factors or biases. For example, clinical signs such as coughing have also been associated with high bacterial counts in the tracheal wash samples (Christley et al., 2001) and mild equine asthma based on BALF cytology (Bedenice et al., 2008). Furthermore, the lack of clinical signs secondary to experimental EHV-2 infection in horses following immunosuppression with dexamethasone or natural infection in foals suggest that EHV-2 is unlikely to cause clinically significant respiratory disease in horses (Dunowska et al., 2011; Fortier et al., 2013).

The likelihood of detecting EHV-5 genome in respiratory samples increased between May and October. Most horses arrived at the track in early April and they may have been re-infected by commingling with other horses (approximately 1200 housed in 17 barns) over time or latent infection resulted in reactivation of the virus following stressful events such as racing throughout the season.

In the present study, the frequency of EHV-2 genome detection in BALF (13 % [4/31]) was within the range reported in healthy athletic horses (0% [0/14] – 36 % [9/25]) (Fortier et al., 2009a; Houtsma et al., 2015). But detection frequency of EHV-5 in BALF (17 %; 5/30) appears higher than previously reported (0% [0/25) – 9% [4/47]) (Fortier et al., 2009a, b; Houtsma et al., 2015). The frequency of detection of ERBV in BALF (3% [1/31]) was comparable to a previous report (7% [1/14]) (Houtsma et al., 2015). The only horse with detectable ERBV genome in BALF in the present study had also viral genome detected in both tracheal and nasal secretions. Contamination of the BAL tube with nasopharyngeal and tracheal secretions during the procedure may have resulted in a false positive test, although 20/31 horses had detectable ERBV in the nasopharynx and/or tracheal secretions but only one horse had detectable ERBV in BALF. Taken together, these findings add to the body of evidence indicating that detection of EHV-2, EHV-5 and ERBV genome by qPCR is common in respiratory samples collected from clinically healthy racehorses.

Virus genome from EHV-1 was not detected from respiratory samples in the present study and only one sample had detectable EHV-4. This was expected because horses had just raced and were not showing clinical signs of respiratory disease. Furthermore, the facility requires proof of vaccine against EHV-1/EHV-4 within 6 months before any horse is allowed on the premises. Alphaherpesviruses such as EHV-1 and EHV-4 are well known respiratory pathogens causing clinical signs such as fever and serous nasal discharge (Gilkerson et al., 2015; Pusterla et al., 2017). However, EHV-1 and EHV-4 may also be detected in respiratory secretions of clinically healthy horses (Fortier et al., 2009a; Doubli-Bounoua et al., 2016) but shedding rate is typically low (Back et al., 2015a; Pusterla et al., 2017). The single horse with detectable EHV-4 in a nasopharyngeal sample had just won the race and had normal BALF cytology. Horses with clinical signs of acute respiratory infection such as fever, cough, nasal discharge, decreased feed intake and lethargy are often diagnosed with viral respiratory diseases (equine influenza, EHV-1, EHV-4) or strangles (*Streptococcus equi* subspecies *equi*) (Mumford et al. 2003, Pusterla et al., 2017) but in 25–74 % of cases, an etiologic agent is not identified (Pusterla et al., 2013).

Because qPCR can detect as little as 2.6–5 copies of the targeted virus genome (Hue et al., 2014), it is important to evaluate not only detection of virus genome but also, quantity of virus and compare both to health outcomes. Detection of ERBV and virus load in nasopharyngeal samples was associated with increased neutrophil proportion in BALF. Only two tracheal samples and one BALF had detectable ERBV therefore, it was not possible to examine potential association with BALF cytology. An association between ERBV in nasal secretions and BALF neutrophilia is concerning because the latter has been linked to poor performance (Ivester et al., 2018). However, BALF neutrophilia in racehorses is also strongly associated with exposure to respirable dust (Ivester et al., 2018). Interestingly, those horses with detectable ERBV had significantly higher respirable dust exposures. It is possible that dust exposure affects both BAL cytology and the presence of ERBV and is the true cause of the relationship observed between BAL neutrophil proportions and the detection of ERBV. This potential confounding effect is further supported by the changes induced when respirable dust exposure was included in the statistical model of the effect of ERBV upon BALF neutrophil proportions. Likewise, the current study did not find an association between ERBV in nasal secretions and performance, but the sample size may have been too small to detect an effect in what is a complex and multifactorial outcome. Another study detected no association between ERBV presence or load in nasal secretion and BALF neutrophilia in mild asthmatic horses but ERBV was only detected in two out of 24 horses (Houtsma et al., 2015). The present study detected an association between increased neutrophil proportion in BALF and EHV-5 detection and load in tracheal but not nasal samples. However, EHV-5 detection and load in BALF was associated with lower neutrophil proportion in BALF. These findings are consistent with other studies reporting no association between BALF neutrophilia and EHV-5 detection or load in tracheal wash or BALF (Fortier et al., 2009a) or an increased likelihood of detecting EHV-5 in BALF from healthy controls as opposed to asthmatic horses (Fortier et al., 2009b). Interestingly, the present study found an association between EHV-2 detection and load in nasopharyngeal samples and reduced mast cell proportions in BALF. Again, this type of association may be a spurious finding due to confounding variables or indicates a potential protective effect of gammaherpesviruses on airway health. Similar controversy exist regarding the role of herpesviruses in childhood asthma (Meel et al., 2020). Again, we should be cautious when interpreting the association between BALF cytology and viruses because few BALF samples had detectable viruses. Unlike ERBV, the effect of gammaherpesviruses upon BAL cytology did not seem to be confounded by environmental exposures.

Severe equine asthma exacerbation is associated with exposure to increased levels of airborne pollen (Costa et al., 2006). In-vitro studies using equine tracheal mucosa and respiratory epithelial cells demonstrated that pollen-derived proteases may disrupt airway epithelium integrity and increase infection rate of epithelial cells by the alphaherpesvirus EHV-1 (Van Cleemput et al., 2019). It is possible that pollen exposure in the current study may have contributed to airway inflammation and infection of the respiratory tract by gammaherpesvirus EHV-2 and EHV-5. Airborne pollen contains high levels of β-glucan (Rylander et al., 1999) but exposure to respirable β-glucan was not associated with EHV-2 or EHV-5 in respiratory samples in the current study.

We chose to sample nasopharynx and trachea using guarded cytology brushes for two reasons: 1) to avoid contaminating the specimen by the endoscope as it passes through the respiratory tract prior to reaching the point of sampling; 2) to sample a similar surface area of the respiratory epithelium with no dilution in order to compare virus load between nasopharynx and trachea. We should be careful when comparing studies that used different sampling methods and sites. For example, nasopharyngeal swabs are more sensitive than nasal swabs for the detection of influenza virus in adult humans (Spencer et al., 2019) and test accuracy depends on the respiratory site sampled as well as the type of virus detected (Loens et al., 2009).

In conclusion, we observed that ERBV, EHV-2 and EHV-5 are commonly detected in the upper airways of healthy racehorses and less frequently detected in BALF. However, we did not identify any link between virus presence or load and clinical signs of respiratory disease or performance. The association between airway inflammation and viruses detected at different level of the respiratory tract was variable. Further studies are needed to determine if ERBV and gammaherpesviruses play a role in the pathophysiology of equine asthma. Many factors determine respiratory health and future studies investigating

the role of subclinical viral infection upon equine asthma should consider environmental exposures.

Declaration of Competing Interest

The authors have no conflict of interest to disclose.

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ORIGINAL ARTICLE - RESEARCH

WILEY

Evaluation of the airway mechanics of modified toggle laryngoplasty constructs using a vacuum chamber airflow model

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Abstract

Objective: To evaluate the airway mechanics of modified toggle LP constructs in an airflow chamber model and compare these to the airway mechanics of standard LP constructs.

Study design: Ex-vivo experimental study.

Sample population: Fifty-one equine cadaveric larynges.

Methods: Bilateral LP constructs were performed using a modified toggle ($n = 23$) or a standard ($n = 21$) LP technique. Constructs were tested in an airflow model before and after cyclic loading which was designed to mimic postoperative swallowing. The cross-sectional area (CSA), peak translaryngeal airflow (L/s), and impedance ($cmH_20/L/s$) were determined and compared between LP constructs before and after cycling.

Results: The mean CSA of the rima glottidis of the modified toggle LP constructs was 15.2 ± 2.6 cm^2 before and 14.7 ± 2.6 cm^2 after cyclic loading, and the mean CSA of the rima glottidis of the standard LP constructs was 16.4 ± 2.9 cm^2 before and 15.7 ± 2.8 cm^2 after cyclic loading. The modified toggle LP constructs had similar peak translaryngeal impedance before and after cyclic loading ($p = .13$); however, the standard LP constructs had higher peak translaryngeal impedance after cyclic loading ($p = .02$).

Conclusion: The modified toggle and standard LP constructs had comparable airway mechanics in an ex-vivo model.

Clinical significance: Further investigation is warranted to determine the extent to which the modified toggle LP technique restores normal airway function in horses with RLN.

1 | INTRODUCTION

Recurrent laryngeal neuropathy (RLN) is a common cause of poor performance in exercising horses.[1–3] Affected horses develop inspiratory noise, increased airway resistance, hypoxemia, hypercapnia, metabolic acidosis, and exercise intolerance.[4–7] Laryngoplasty (LP), combined with cordectomy or ventriculocordectomy, is considered the gold standard treatment in horses diagnosed with RLN[8] as the procedure can restore airway function in horses with experimentally induced RLN to that similar to presurgical baseline values.[6,7,9,10] Postoperative loss of arytenoid abduction and reduced cross-sectional area (CSA) of the rima glottidis is a frequent complication of the LP procedure.[11] Sudden and complete loss of arytenoid abduction has been reported in up to 11% of horses following the LP procedure,[11,12] and gradual loss of arytenoid abduction over the first 6 weeks after surgery has been reported in 76–95% of horses.[12,13] Arytenoid abduction loss may partially explain the variable success rates reported for the standard LP, particularly in racehorse populations.[12–15]

This progressive loss of arytenoid abduction after surgery has been attributed to partial suture pull-through from the muscular process of the arytenoid cartilage due to cyclic loading of LP sutures associated with coughing and swallowing after surgery.[16] Pharyngeal constrictor muscles reside circumferentially around the larynx and contribute to repeated loading of the arytenoid cartilage and larynx.[16] A LP technique that uses a titanium suture button to secure the suture material to the muscular process of the arytenoid cartilage was recently evaluated in cadaveric equine larynges.[17] The use of a modified toggle LP technique eliminated suture pull-through from the muscular process using an ex-vivo loading model that mimicked postoperative cyclical adductory forces applied to the LP.[17] However, before clinical use of this modified toggle LP technique, it is necessary to evaluate its performance under airflow and inspiratory pressures comparable to those recorded in horses during maximal exertion.

Translaryngeal impedance is an objective measure of airflow obstruction and has been used to compare the effect of different LP techniques to restore normal airflow in horses.[6,7,18,19] Cheetham et al. developed a flow chamber that produces airflow and inspiratory pressures similar to those recorded in horses during maximal exertion.[20] This experimental model can be used to determine the translaryngeal impedance of different LP techniques under conditions of high airflow rates.[19–22] Moreover, this experimental model exposes LP constructs to intermittent airflow in a controlled fashion, thus reducing experimental variability.[19,21–23] The objective of this study was to evaluate the airway mechanics of modified toggle LP constructs in an airflow chamber model and compare these to the airway mechanics of standard LP constructs. We hypothesized that the CSA of the rima glottidis and translaryngeal impedance of the standard and modified toggle LP constructs would remain constant after cyclic loading.

2 | MATERIALS AND METHODS

2.1 | Sample collection and storage

Fifty-one grossly normal larynges attached to the most proximal tracheal rings were obtained with owner consent from horses (age range, 2–25 years; 32 geldings, 15 mares and 4 colts) from various breeds (17 Quarter horses, 7 Standardbreds, 5 Warmbloods, 5 Tennessee Walking horses, 3 Thoroughbreds, 3 American Saddlebreds, and 11 mixed breed) after humane euthanasia for reasons unrelated to the study and unrelated to disease of the respiratory tract. Of these 51 specimens, six were removed from the study and replaced following fracture of the arytenoid cartilage during standard LP construct preparation. A pilot study was conducted before the study to determine the sample size; the sample size calculated was consistent with previous cadaveric ex-vivo mechanical studies of LP.[24–26] Larynges were collected within 5 h of euthanasia, wrapped in gauze soaked in saline (0.9% NaCl) solution, and stored at −20°C until the time of the study.[17] Specimens were thawed for 12 h at room temperature (20°C) before each study phase.[17,19] Specimens were frozen for at least 24 h prior to thawing for any use.

2.2 | Construct preparation

For the main study, the specimens were distributed into five age groups: ≤5 years, ≥6 to ≤10 years, ≥11 to ≤15 years, ≥16 to ≤20 years, and ≥21 to ≤25 years. Within each group, specimens were randomly allocated to either bilateral modified toggle LP technique or bilateral standard LP technique using a computational randomization function (Microsoft Excel, Microsoft Corporation, Redmond, Washington). Prior to LP suture placement, each specimen had both cricoarytenoideus dorsalis muscles removed to facilitate consistent suture placement and the dorsal aspect of the cricoarytenoid joints were incised. This was performed to mimic the clinical scenario in which LP is combined with arthrotomy of the cricoarytenoid joint to induce ankylosis and thereby reduce postoperative loss of arytenoid abduction.[27]

Standard LP constructs were prepared using a common technique.[9] A strand of #5 polyester coated

polyethylene suture (Fiberwire, Arthrex Inc., Naples, Florida) was passed through the cricoid cartilage approximately 2 cm rostral to the caudal border and 1 cm abaxial to its dorsal ridge. The suture was then passed through the left muscular process in a caudomedial to rostrolateral direction ensuring the spine of the muscular process was engaged. A second strand of the same suture material was placed approximately 5 mm lateral to the first suture on the cricoid cartilage and passed in the same direction with the second strand of suture passing approximately 3 mm ventral to the first suture through the muscular process. The suture was then tightened until 90% of maximal abduction was achieved subjectively,[28] and tied with six single throws. The procedure was repeated on the right side of each specimen (Figure 1A).

Modified toggle LP constructs were prepared as previously described.[17] Briefly, a strand of #5 polyester coated polyethylene suture (Fiberwire, Arthrex Inc., Naples, Florida) was passed through the cricoid cartilage as described for the standard LP technique. Then, the apical portion of the muscular process was removed with a No. 10 scalpel blade to create a flat surface. Placement of the suture through the muscular process of the arytenoid cartilage was performed by drilling a 2.7 mm hole through the center of the muscular process. The drill bit was centered within the flat surface and aimed toward the base of the muscular process so that a tract was created from caudodorsal to cranioventral. The strand of suture was threaded through a 12 mm titanium suture button (RetroButton, Arthrex Inc., Naples, Florida) and the button was passed through the drilled hole. A 2.5 mm metallic probe was used to confirm that the titanium suture button had exited the far side of the drill tract. The suture was then pulled tight to lock the toggle on the base of the muscular process, and then tightened until 90% of maximal abduction was achieved subjectively.[28] The suture was tied with six single throws. The procedure was repeated on the right side of each specimen (Figure 1B).

Bilateral ventriculocordectomy was performed in all specimens after LP procedures using a standard technique.[9] Briefly, a Virginia roaring burr (Sontec Instruments, Centennial, Colorado) was introduced into the laryngeal ventricle and rotated until the mucosa was engaged. The ventricle was then everted into the laryngeal lumen by gentle traction on the burr and removed using Metzenbaum scissors. The vocal fold was grasped with Allis tissue forceps and removed after transection of its most dorsal and ventral attachments.

2.3 | Analysis of airflow

Airflow evaluation was performed with a flow chamber as previously described.[20] The flow chamber had an ultrasonic flowmeter inserted between the test box and the vacuum system to measure airflow along with two polytetrafluoroethylene catheters (4.8 mm ID, 6.4 mm OD, 50 cm long) connected to a differential pressure

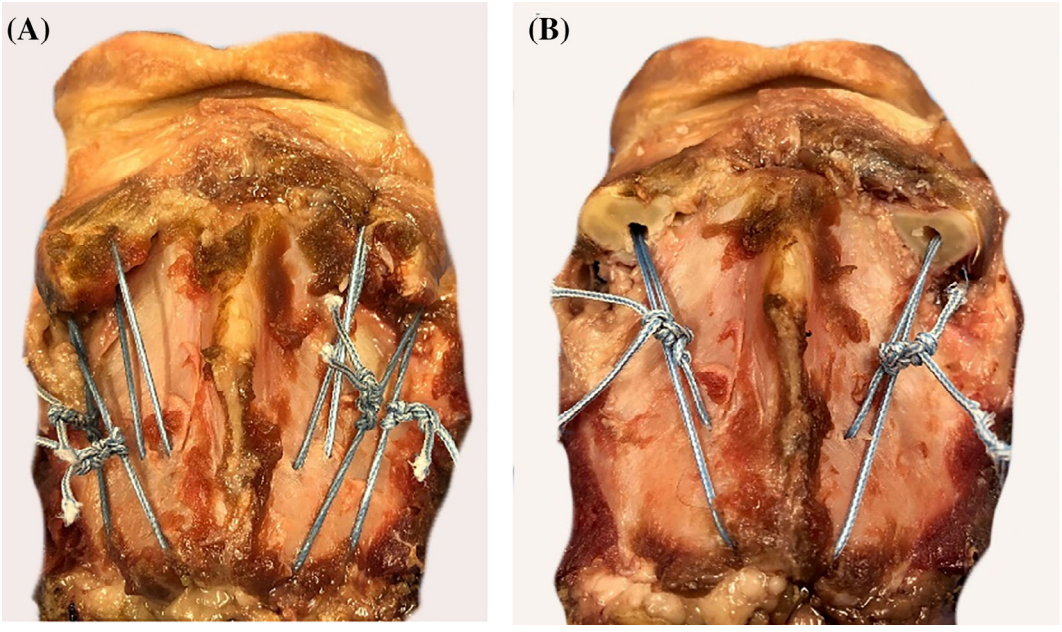

FIGURE 1 Dorsal view of the standard (A) and modified toggle (B) LP constructs. Rostral is at the top of the picture. All specimen had both cricoarytenoideus dorsalis muscles removed and the cricoarytenoid joints disarticulated before suture placement

transducer to measure peak translaryngeal pressure. One of the catheters was inserted in the right side of the test box with its tip positioned rostral to the specimen and the second catheter was inserted through the PVC pipe with the tip positioned 10 cm caudal to the rima glottidis (Figure 2).

Pressure transducer and flowmeter output signals were recorded using a commercial computer software (Pulmonary Mechanics Analyzer, Buxco Electronics, Inc.) at a sampling rate of 100 Hz. Before each experiment, the pressure transducer was calibrated from 0 to 50 cmH_2O using a water manometer and the flowmeter was calibrated between 0 and 60 L/s using a rotameter. Before testing any larynges, the flow chamber was always tested for leaks. Then, the cycling valve of the flow chamber was fully opened, and the vacuums adjusted to achieve a maximal airflow of 50 L/s (48–54 L/s).

Baseline and follow-up airflow evaluations were performed within 4 h of LP construction and 24 h after thawing following cyclic loading respectively. All specimens were exposed to cyclic airflow (2 Hz) to mimic the respiratory frequency of 110–120 breaths/min in horses exercising at 14 m/s.[10] The peak translaryngeal flow (L/s) and pressure (cmH_20) were recorded for at least 30 s and impedance (cmH_20/L/s) was calculated as the ratio of translaryngeal pressure divided by airflow by averaging data from each cycle.

2.4 | Cyclic loading

All larynges were cycled in circumferential loading designed to mimic forces exerted on the LP sutures due to swallowing and coughing.[16] Each specimen was placed in a plastic cradle and a 2.5 cm wide nylon strap was secured around the larynx and cradle with the muscular process centered under the webbing (Figure 3). The ends of the nylon strap were secured to the stationary crosshead/load cell and the servo-hydraulic actuator of a load frame (Instron 880, Instron, Co.). The ends of the strap were displaced by 25 mm in the load-frame, resulting in 40–45 N of load on the prosthetic suture.[17] Larynges were loaded at 2 Hz for

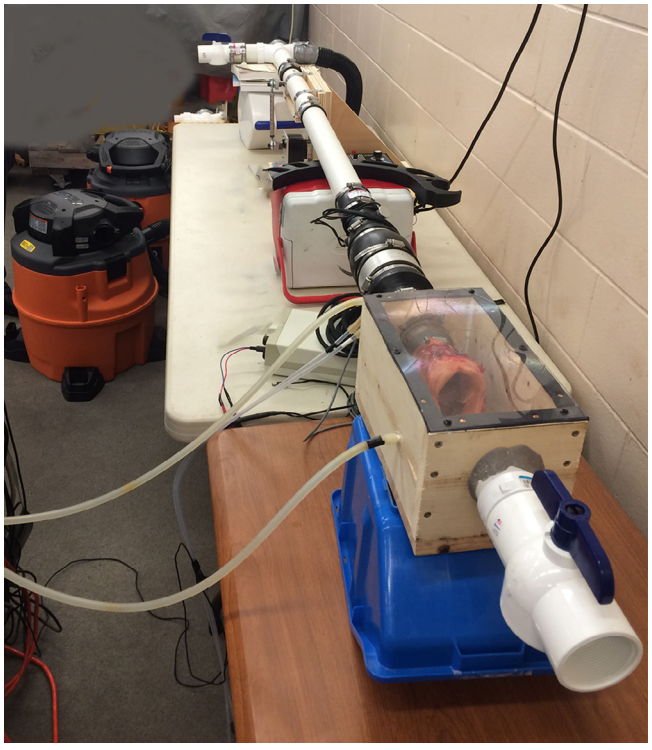

FIGURE 2 Photograph of the experimental setup for airflow testing. Translaryngeal pressure was measured by calculating the difference between pharyngeal pressure and tracheal pressure. Each larynx was placed in the test box and the epiglottis was secured using a singular screw and the tracheal ring was secured using a ring clamp to an adaptor fitted to the PVC pipe prior to testing

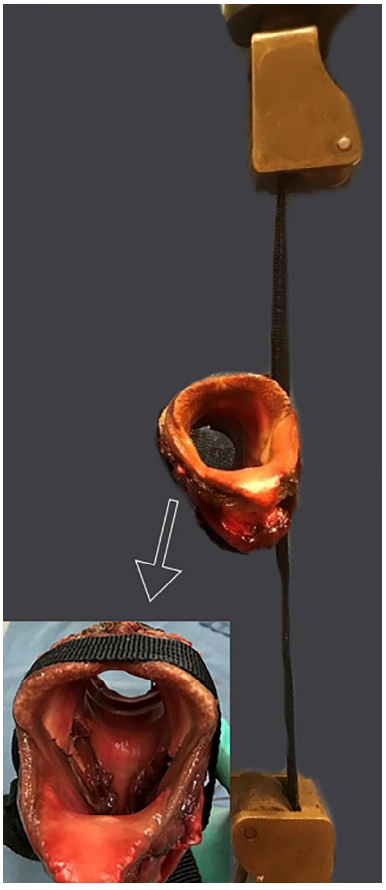

FIGURE 3 Photograph illustrating how the specimens were subjected to cyclic loading on the load frame and how the nylon strap was applied around to the laryngeal specimens and centered over the muscular processes of the arytenoid cartilages (arrow)

10 000 cycles. The number of cycles and load approximate the force applied to the LP sutures due to swallowing for a week postoperatively, which is when the majority of progressive loss of arytenoid abduction occurs.[11,12,16]

2.5 | Cross-sectional area measurement

Photographs were obtained before and after cyclic loading to assess the progressive loss of arytenoid abduction. Each larynx was placed in a customized cradle positioned 15 cm from a digital camera (iPhone 7, Apple, Cupertino, California). The rima glottidis CSA of each larynx was measured by the same investigator using the same software (Image J version 1.51k, National Institutes of Health, Bethesda, Maryland) after calibration of image size using a 2.5 cm × 2.5 cm template that was fixed to the rostral aspect of the cradle.[25] To determine the CSA, a line was digitally traced around the inside of the rima glottides to the top of the vocal fold (Figure 4) as previously described.[29,30] Rima glottidis CSA (cm^2) was measured three times and the mean value determined for each specimen.[25]

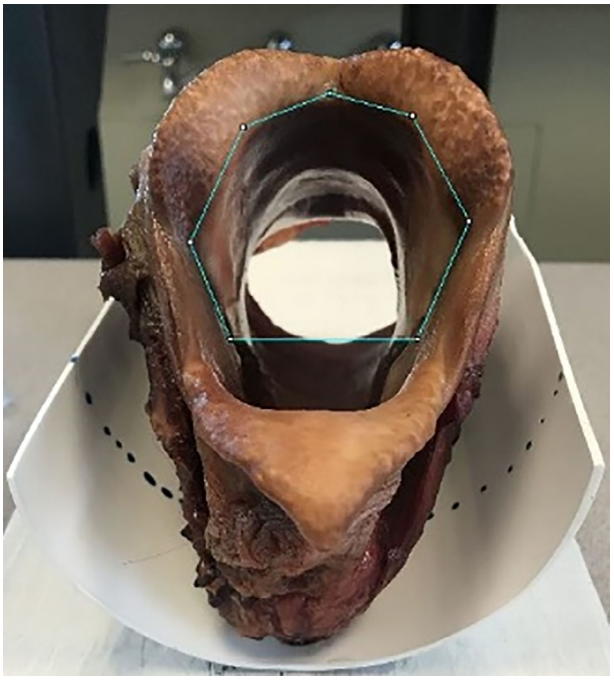

FIGURE 4 Photograph of specimen placed in the larynx cradle to show how measurement of rima glottidis cross-sectional area was obtained. A line was drawn along the lumen of the rima glottidis with the lower bound at the level of the top of the vocal fold

2.6 | Statistical analysis

The Shapiro–Wilk test, skewness, kurtosis, and probability plots were used to assess the distribution of rima glottidis CSA of the LP constructs before and after cyclic loading. The CSA data (cm^2) was normally distributed, so a paired t- test was used to compare the CSA of each LP technique. Age differences in the CSA of the larynges data were analyzed using Tukey's HSD test. As the airway mechanics data did not have normal distributions, a Mann–Whitney *U* test was used for maximal inspiratory airflow (L/s) and translaryngeal impedance (cmH_20/L/s) for each LP technique. Construct failures were compared by a one-sided Fisher Exact test. Analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, North Carolina), with $p < .05$ considered significant.

3 | RESULTS

3.1 | Cross-sectional area of the specimens

The mean rima glottidis CSA of the modified toggle LP constructs was 15.2 ± 2.6 cm^2 (coefficient of variation: 17.1%) before and 14.7 ± 2.6 cm^2 (coefficient of variation: 17.7%) after cyclic loading, whereas the mean CSA of the rima glottidis of the standard LP constructs was 16.4 ± 2.9 cm^2 (coefficient of variation: 17.7%) before and 15.7 ± 2.8 cm^2 (coefficient of variation: 17.8%) after cyclic loading. While cyclic loading resulted in a slight loss of arytenoid abduction in all 45 LP constructs, it was not significant in either the modified toggle LP constructs ($p = .50$) or the standard LP constructs ($p = .46$). There was no effect of age on the difference in CSA of the rima glottidis ($p > .50$).

3.2 | Airway parameters

All LP construct sutures remained intact during the airflow testing and none of them experienced axial collapse of the arytenoid cartilages during testing; however, one LP construct was noted to have a tear in the cricothyroid ligament during follow-up airflow testing, so it was excluded from the final analysis, leaving a total of 44 specimens with airway mechanics data (23 modified toggle and 21 standard LP constructs).

The peak inspiratory airflow was similar before (median = 50.6 L/s; range = 48.4–51.6 L/s) and after (median = 50.5 L/s; range = 48.8–52.1 L/s) cyclic loading of the modified toggle LP constructs ($p = .18$). The peak translaryngeal impedance was also similar before

(median = 0.22 $cmH_20/L/s$; range = 0.13–0.40 $cmH_20/L/s$) and after cyclic loading (median = 0.22 $cmH_20/L/s$; range = 0.16–0.57 $cmH_20/L/s$) of the modified toggle LP constructs ($p = .13$).

The peak inspiratory airflow was similar before (baseline median = 50.7 L/s; range = 48.6–52.3 L/s) and after (follow-up median = 50.6 L/s; range = 49.4–51.5 L/s) cyclic loading of the standard LP constructs ($p = .90$). However, there was a slight difference in the distributions of peak translaryngeal impedance before (baseline median = 0.21 $cmH_20/L/s$; range = 0.12–0.35 $cmH_20/L/s$) and after (follow-up median = 0.21 $cmH_20/L/s$; range = 0.14–0.45 $cmH_20/L/s$) cyclic loading of the standard LP constructs ($p = .02$).

4 | DISCUSSION

The main finding of our study was that the modified toggle and standard LP constructs had comparable airway mechanics in an ex-vivo model. The study design aimed to mimic the clinical scenario in which the LP sutures placed in horses are subjected to adductory forces associated with swallowing for slightly more than 1 week, which is when postoperative loss of arytenoid abduction has been reported to occur,[3,31–34] and subsequently exposed to intermittent airflow during exercise. Both LP techniques had negligible similar progressive loss of arytenoid abduction after cyclic loading; however, 6 of 27 specimens undergoing the standard LP technique had breakage of the muscular process and had to be removed from the study. Breakage of the muscular process is a reported intraoperative complication of the standard LP technique in clinical cases[8] and the fact that no modified LP constructs (0 of 23; $p = .0077$) had to be removed from the study due to this complication may represent an advantage of the modified toggle LP technique. Based on a pilot study we performed (data not presented here) investigating the effect of freeze thaw cycles on tissue quality, it is unlikely the observed cartilage failure was linked to processing procedures. In a previous study, cyclic loading of the standard LP constructs, which were built with only one prosthetic suture, resulted in greater loss of rima glottidis CSA when compared to modified toggle LP constructs.[17] In the current study, the standard LP constructs were prepared with two prosthetic sutures, which is common practice during the LP surgery, and likely increases the pull-out strength of the arytenoid cartilages.[24]

The main determinants of translaryngeal impedance include resistance, elastance, and inertance of the tissues. The most important component of impedance is resistance, which is largely influenced by the diameter of the respiratory tract.[35,36] According to a computational modeling study, the necessary degree of arytenoid cartilage abduction during the LP procedure should be approximately 88% of the maximal CSA to restore airway patency at maximal exercise in racehorses.[28] However, other investigators have shown that 80–90% of maximal arytenoid abduction following the LP procedure is unnecessary to restore inspiratory flow limitations in horses with experimentally induced RLN and exercising at 100% of maximal heart rate.[4,6,7,9,10] Moreover, National Hunt Thoroughbred racehorses with 45–90% of the maximal arytenoid cartilage abduction following LP had similar performance after surgery.[14] It has been proposed that fixing the arytenoid in a moderately abducted position (approximately 50–60%) and thus preventing dynamic collapse is more important than obtaining maximal abduction after LP.[13,14] In this study, the mean CSA of the rima glottidis decreased by 3.3% among the modified toggle LP constructs and by 4.3% among the standard LP constructs following cyclic loading. Therefore, we think the small reduction in CSA of the rima glottidis experienced by all LP constructs is unlikely to be clinically significant. This is further supported by the airflow evaluation, which did not significantly change after cyclic loading despite this small change in the CSA in all the LP constructs. Loss of abduction would be even smaller in clinical cases due to the LP procedure only being performed on one side.[8]

The LP constructs were assessed by subjecting specimens to negative pressure and airflow using the model described by Cheetham[20] and used by others.[18,19,21–23] In our study, the modified toggle LP constructs had similar peak translaryngeal impedance before and after cyclic testing, whereas the standard LP constructs had a slight increase in peak translaryngeal impedance after cyclic testing. We suggest that the slightly higher peak translaryngeal impedance after cyclic loading in the standard LP constructs is unlikely to be clinically significant as the range of peak translaryngeal impedance measurements among the LP constructs was similar to those reported in horses with adequate postoperative arytenoid cartilage abduction.[10] We suggest that the modified LP technique can handle the forces applied to the construct in the immediate postoperative period, including exposure to airflow and inspiratory pressures similar to those recorded in exercising horses, and is as effective in restoring airway mechanics as the standard LP technique.

In our previous and current study, the use of the modified toggle LP technique prevented breakage or suture pull-through at the muscular process,[17] which is a common mechanism for early postoperative loss of arytenoid abduction.[8,12,16] Other recent research efforts have aimed to mitigate postoperative "relaxation" of the

prosthetic suture have focused on the use of suture buttons to anchor or toggle the prosthetic suture to the cricoid cartilage.[26] An ex-vivo study showed that suture material passed through metallic buttons placed on the ventral aspect of the cricoid cartilage reduced suture rostroventral slipping, and eliminated suture pull out the cricoid cartilage.[26]

Limitations of this study are inherent to its ex-vivo nature. First, cadaveric larynges have no active muscular function and cannot resist dynamic collapse in the same way as a larynx in-vivo, which could affect arytenoid abduction and impedance. Therefore, the LP procedure was performed on both sides of each specimen, which does not mimic the clinical scenario. Second, the model does not mimic the nasopharynx; therefore, the potential ability of both LP techniques to prevent the dynamic collapse of the nasopharyngeal structures (i.e., medial deviation of the aryepiglottic folds) during inspiration remains unknown. Third, to acquire the necessary number of larynges for the study cadaver specimens of all ages and breeds were collected. However, age groups had similar CSA of laryngeal specimens after cyclic loading, therefore cartilage retention of the LP prostheses. Fourth, the modified LP technique was easily performed ex-vivo and did not require more time than the standard LP to perform; however, this was not objectively measured. Fifth, concerns regarding the feasibility of performing this technique in-vivo remain to be investigated; however, the authors have performed this procedure in intact cadaver specimens successfully with a similar surgical approach and incision size used for a standard LP. Sixth, the flow chamber was set to achieve a maximal airflow of 50 L/s which has been reported in horses exercising at 14 m/s[9,10]; however, other studies[37,38] have shown that exercising racehorses may develop higher maximal airflow rates (>75 L/s). Exposure of the LP constructs to a higher maximal airflow may have the potential to impact the results of our study.

In conclusion, the modified toggle and standard LP constructs had comparable airway mechanics in an ex-vivo model. The results of this study represent a critical step in the validation of our modified LP technique prior to wide clinical use.

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CONFLICT OF INTEREST

The authors report no conflict of interest in regards to this study.

AUTHOR CONTRIBUTIONS

SMG, DVM, DACVS-LA: study design, specimen collection, preparation, and construction, data collection, analysis, and interpretation, manuscript draft and revisions, and final approval of the manuscript; SDG, DVM, MS, DACVS-LA, DACVSMR-EQ: study design, specimen construction, data analysis and interpretation, manuscript draft and revisions, and final approval of the manuscript; LLC, DVM, PhD, DACVIM-LA: study design, data collection and interpretation, and final approval of the manuscript; GPH, PhD: study design, data collection and interpretation, and final approval of the manuscript; RMK, MS: study design, data collection, and final approval of the manuscript; AMM, DVM, MS, PhD, DACVS-LA: study design, data interpretation, and final approval of the manuscript; MCS, BVSc, PhD, FACVSc, DACVSMR-EQ: study design, data interpretation and final approval of the manuscript; DJS, PhD: preliminary and final data analysis and interpretation, manuscript draft and revisions, and final approval of the manuscript.

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ORIGINAL ARTICLE

Pharmacokinetics of thiamine (vitamin B1) in adult horses after administration of three single intravenous doses

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Abstract

Thiamine is a vital co-factor for several anti-inflammatory and antioxidant processes that are critical for mitigation of sepsis-associated inflammation, but pharmacokinetic (PK) analysis has not been reported in horses. We hypothesized that IV thiamine hydrochloride (TH) at increasing dosages would result in corresponding increases in plasma thiamine concentrations without causing adverse effects. A randomized cross-over study was performed in 9 healthy horses that each received TH at 5, 10, and 20 mg/kg IV. Blood was collected immediately prior to drug administration and at several time points thereafter. High-performance liquid chromatography with mass spectrometry was used to quantify thiamine concentrations at each time point. Non-compartmental PK methods showed that IV TH resulted in supraphysiologic plasma concentrations with a short half-life (0.77–1.12 h) and no adverse clinical signs were observed. The terminal rate constant decreased as the dosage increased ($p < .0001$) and clearance significantly decreased at the 20 mg/kg dosage ($p = .0011$). The area under the curve (AUC) increased in a non-linear fashion. These findings suggest that thiamine follows non-linear elimination kinetics in horses, which is likely due to saturation of renal elimination. Future studies are needed to identify therapeutic plasma concentrations and develop thiamine dosing recommendations for horses.

KEYWORDS
horse, metabolic, pharmacokinetics, thiamine, vitamin B1

1 | INTRODUCTION

Sepsis continues to represent a common cause of morbidity and mortality in neonatal foals and adult horses (Arroyo et al., 2017; Macias-Rioseco et al., 2020; Peek et al., 2006; Wong et al., 2018). The dysregulated host response to microbial invasion, termed systemic inflammatory response syndrome (SIRS), can lead to rapid deterioration in the face of pathogen control. Current therapy focuses on broad-spectrum antimicrobial treatment and fluid resuscitation, but this is often inadequate in reversing the catastrophic effects of SIRS. During SIRS, pro-inflammatory cytokines are produced in excess and trigger release of acute phase proteins from the liver. Collectively, these inflammatory mediators act on endothelial cells to cause vasodilation and increased vascular permeability, which lead to tissue hypoxia and lactic acidosis (Joffre et al., 2020; Peters et al., 2003). Activation of the immune system is accompanied by a complex chain of redox events that include generation of reactive oxygen species (ROS), particularly by neutrophils (Woodfin et al., 2016). Thiamine (Vitamin B1) is a vital co-factor for many cellular metabolic processes, including aerobic metabolism and ROS clearance (Costa et al., 2014; Frank et al., 2007). Thiamine deficiency is common in septic humans and is associated with refractory

lactic acidosis and death (Woolum et al., 2018). Thiamine supplementation improves neutrophil phagocytosis in sheep (Olkowski et al., 1990) and reduces the risk of renal damage in septic humans (Moskowitz et al., 2017). Furthermore, thiamine administration within 24 h of admission in human patients with sepsis is associated with improved lactate clearance and survival compared to controls (Woolum et al., 2018).

Depletion of endogenous thiamine and ascorbic acid (Vitamin C) has been documented in septic humans, (Attaluri et al., 2018; Donnino et al., 2010; Lima et al., 2011; Schorah et al., 1996; Wilson, 2009) and endogenous serum cortisol concentrations are often inadequate for the degree of illness (Marik, 2007; Soni et al., 1995). Therefore, administration of thiamine hydrochloride (TH), ascorbic acid, and hydrocortisone has been shown to dampen inflammation and improve survival in septic humans (Kim et al., 2018; Marik et al., 2017). This combination therapy, termed "metabolic resuscitation," might also benefit septic horses, but data are lacking. In horses, endogenous ascorbic acid depletion has been reported after IV lipopolysaccharide (LPS) infusion in the majority of subjects, (Anderson et al., 2020) and critical illness-related cortisol insufficiency is common in septic horses; (Anderson et al., 2020; Hart & Barton, 2011; Stewart et al., 2019) however, it is unknown whether or not thiamine depletion occurs in sick horses.

Thiamine hydrochloride has been used as adjunctive treatment for several conditions in the horse, including neurological and renal diseases, but specific therapeutic effects and therapeutic plasma thiamine concentrations have not been determined (Holbrook et al., 2007; Loew, 1973; Steele, 1948; Wilkins et al., 1994). In addition, an appropriate dosage of TH for use in the horse has not been established. Finally, TH has been reported to cause anaphylactic reactions in ruminants and humans when administered IV, (Cebra & Cebra, 2004; McLaughlin et al., 2003; Stephen et al., 1992; Thomson et al., 2019) but adverse effects of IV TH administration have not been adequately studied in horses. Therefore, the objectives of the study reported here were to evaluate the pharmacokinetic profile of thiamine in healthy adult horses after single IV dose administration at three dosage levels (5, 10, and 20 mg/kg) and to investigate the potential adverse effects after IV TH administration.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

Nine adult horses from a university teaching herd were determined to be healthy based on physical examination, complete blood count (CBC), and serum biochemical analysis (SBA). A repeated Latin square design was used to ensure that each horse received each drug dosage (5, 10, and 20 mg/kg). Horses were randomly assigned a number (1 through 9) and divided into groups of 3. Each dosage was randomly assigned to each horse. Each of the 3 treatment trials was staggered over the course of 3 days. All horses were fed free-choice grass hay during each treatment trial and turned out on pasture between trials. The horses consisted of 6 geldings and 3 mares that ranged in age from 13 to 18 years, with a mean of 16 ± 2 years. Five breeds were represented, including 3 Quarter Horses, 2 Thoroughbreds, 2 Warmbloods, 1 Saddlebred and 1 Standardbred. The horses weighed 445–641 kg and were weighed immediately prior to the start of each treatment trial to ensure accurate dosing. All horses gained weight over the course of the study, with a mean weight gain of 32 ± 21 kg from the first to the last treatment trial. This was attributed to access to lush pasture grass between treatment trials. Demographic data for individual horses are presented in Table S1. All procedures were approved by the Institutional Animal Care and Use Committee at Purdue University.

2.2 | Drug administration

Thiamine hydrochloride (Neogen Vet, 500 mg/ml, Lexington, KY, USA) was administered IV at 5, 10, and 20 mg/kg. These dosages were chosen based on anecdotal reports in large animals and extrapolation from human sepsis studies (Apley, 2015; Maiti et al., 1990; Marik et al., 2017). Drug was administered over a 10 min period for each treatment trial. A washout period of ≥1 week was chosen since this was >7 times the expected elimination half-life of approximately 5 h (Smithline et al., 2012; Tallaksen et al., 1993). Bilateral IV jugular catheters were aseptically placed the night before the start of each treatment trial. One jugular vein was used for drug administration and the other for blood collection, alternating sides in subsequent trials. Heparinized blood samples were collected immediately prior to drug administration (T0; baseline) and at 5, 10, 15, 20, 30, 45, 60, and 90 min and 2, 4, 6, 8, 10, 12, 24, and 48 h. The T5m sample was collected 5 min after the end of the 10 min drug administration. Blood tubes were light-protected and stored on ice prior to centrifugation. Plasma was collected following centrifugation at 1,300 g for 5 min within 2 h of collection and stored at −80°C until analysis (Tashirova et al., 2013). For each horse during each trial, the jugular catheter used for drug administration was removed within 2 h of drug administration and the catheter used for blood collection was removed immediately following blood collection at T24 h. All T48 h blood samples were collected via jugular venipuncture.

2.3 | Adverse effects

Horses were continually monitored for the first 12 h of each treatment trial, with complete physical examinations performed at T0 (baseline), 6, 12, 24, and 48 h. A CBC and SBA were performed immediately prior to the start of the study to ensure overall systemic health prior to the start of the study. Subjective assessments were made regarding changes in appetite, behavior, and fecal consistency. Jugular catheter insertion sites and jugular veins were assessed for swelling, heat, and pain.

2.4 | Sample analysis

2.4.1 | Sample preparation

Frozen horse plasma was stored at −80°C until analysis. The extraction protocol was based on modifications from Al-Attas and McCann (Al-Attas et al., 2012; McCann et al., 2017). Each sample was thawed and a 0.1 ml aliquot was transferred to microcentrifuge tube for extraction. Isotopically labeled ($^{13}C_3$) thiamine was used as an internal standard and for quantification of endogenous thiamine in each sample (Toronto Research Chemicals, North York, ON). Each sample aliquot was spiked with 100 ng of $^{13}C_3$-thiamine prior to the extraction process. To extract each sample, 25 µl of 10% trichloroacetic acid (TCA) was added and the sample placed on ice for 15 min. Samples were then vortexed for 3 min and centrifuged at 15,000 g for 10 min to precipitate proteins. The supernatant was collected and pellet discarded. The supernatants were stored at −20°C until ready for analysis on a LC/MS/MS system. At the time of analysis, each sample was diluted 20-fold with a solution of 50% water/50% acetonitrile, vortexed, and transferred to an autosampler vial.

2.4.2 | HPLC/MS-MS analysis

An Agilent 1260 Rapid Resolution liquid chromatography (HPLC) system coupled to an Agilent 6470 series triple quadrupole mass spectrometer (MS/MS) was used to analyze thiamine (Agilent Technologies, Santa Clara, CA). A Waters Acquity BEH HILIC (2.1 mm × 100 mm, 1.7 µm) column was used for HPLC separation (Water Corp, Milford, MA). The buffers were (A) acetonitrile: isopropyl alcohol:200 mM ammonium formate at pH 3 (90:5:5 v/v) and (B) water: acetonitrile:200 mM ammonium formate at pH 3 (90:5:5 v/v). The linear HPLC gradient was as follows: time 0 min, 0% B; time 1 min, 0% B; time 7 min, 40% B; time 8 min, 90% B; time 11 min, 0% B; and time 15 min, 0% B. The flow rate was 0.3 ml/min. Multiple reaction monitoring was used for MS analysis. Data were acquired in positive electrospray ionization (ESI) mode. The calibration curve ranged from 100 to 0.001 µg/ml. Quantitation was based on the ion transition of thiamine 265.5→122.4 and $^{13}C_3$-thiamine 268.5→122.4 and qualifier ions 265.5→144.4 and 268.5→147.4. Agilent Masshunter Quantitative Analysis software was used for data analysis (version 8.0).

2.5 | Statistical analysis

Data are expressed as mean ± standard deviation (range). To account for endogenous plasma thiamine, for each horse at each dosage level (5, 10, and 20 mg/kg), plasma thiamine concentrations were normalized by subtracting the endogenous thiamine concentration at time 0, prior to TH administration. Any negative values were entered into the analysis as 0 µg/ml. For each IV TH dosage level, non-compartmental pharmacokinetic analysis was performed using commercially available software (Phoenix WinNonLin 8.1, Certara, Princeton, NJ). Calculated parameters included the terminal rate constant (λ_z), terminal half-life ($t_{1/2}$), concentration at time 0 (C_0), observed area under the curve (AUC_{obs}), area under the curve extrapolated to infinity ($AUC_{0-\infty}$), percent of $AUC_{0-\infty}$ extrapolated ($AUC_{\%extrap}$), volume of distribution by the area method (V_z), clearance (Cl), observed area under the moment curve ($AUMC_{obs}$), area under the moment curve extrapolated to infinity ($AUMC_{0-\infty}$), percent of $AUMC_{0-\infty}$ extrapolated ($AUMC_{\%extrap}$), mean residence time (MRT), and volume of distribution at steady state (V_{ss}). To determine whether thiamine is eliminated in a linear fashion in the horse or is affected by dosage, λ_z and Cl were each compared between dosage levels. First, data normality was assessed using the Kolmogorov–Smirnov test. Then, each parameter (λ_z and Cl) was compared between dosages using a repeated measures ANOVA and Tukey's *post hoc* test for multiple comparisons. Increases in $AUC_{0-\infty}$ with dosage were also visually assessed for linearity in individual horses. Statistical comparisons were performed using commercially available software (Prism 9; GraphPad Software, LLC., San Diego, CA). Significance was set at $p < .05$.

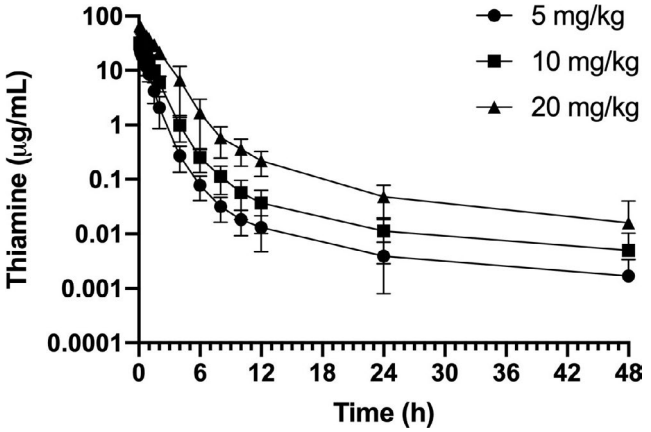

FIGURE 1 Plasma concentration–time relationship of thiamine hydrochloride after 5, 10, and 20 mg/kg IV dosing in healthy horses ($n = 9$)

3 | RESULTS

3.1 | Pharmacokinetics

Plasma thiamine concentration vs. time plots are presented in Figure 1. Pharmacokinetic parameters for horses administered TH at 5, 10, and 20 mg/kg IV are presented in Tables 1–3. Individual horse plasma thiamine concentrations and pharmacokinetic parameters for all three dosages assessed are presented in Tables S2 and S3, respectively.

To determine whether thiamine kinetics are linear in horses, λ_z and Cl were assessed for changes between dosage levels. λ_z significantly decreased as dosage increased ($p < .0001$) as seen

TABLE 1 Non-compartmental pharmacokinetic parameters for thiamine hydrochloride in healthy horses (n = 9) after a single dosage of 5 mg/kg

Parameter	Mean	SD	Min	Max
λ_z (h^{-1})	0.905	0.055	0.803	0.964
Half-life (h)	0.77	0.05	0.72	0.86
C_0 (μg/ml)	29.81	4.26	25.51	38.79
AUC_{obs} (h*μg/ml)	23.82	4.97	16.90	30.56
$AUC_{0-\infty}$ (h*μg/ml)	23.83	4.97	16.91	30.56
$AUC_{\%extrap}$ (%)	0.02	0.02	0.00	0.08
V_z (ml/kg)	240.0	41.4	179.5	309.0
Cl (ml/kg/h)	218.4	46.4	163.6	295.8
$AUMC_{obs}$ (h^2*μg/ml)	24.49	9.56	13.69	38.84
$AUMC_{0-\infty}$ (h^2*μg/ml)	24.62	9.62	13.75	38.99
$AUMC_{\%extrap}$ (%)	0.51	0.23	0.19	0.82
MRT (h)	1.00	0.20	0.69	1.33
V_{ss} (ml/kg)	212.7	31.4	174.5	259.9

Abbreviations: λ_z, terminal rate constant; terminal half-life ($t_{1/2}$); $AUC_{\%extrap}$, percent of $AUC_{0-\infty}$ extrapolated; $AUC_{0-\infty}$, area under the curve extrapolated to infinity; AUC_{obs}, observed area under the curve; $AUMC_{\%extrap}$, percent of $AUMC_{0-\infty}$ extrapolated; $AUMC_{0-\infty}$, area under the moment curve extrapolated to infinity; $AUMC_{obs}$, observed area under the moment curve; C_0, concentration at time 0; Cl, clearance; MRT, mean residence time; V_{ss}, volume of distribution at steady state; V_z, volume of distribution by the area method.

TABLE 2 Non-compartmental pharmacokinetic parameters for thiamine hydrochloride in healthy horses (n = 9) after a single dosage of 10 mg/kg

Parameter	Mean	SD	Min	Max
λ_z (h^{-1})	0.774	0.063	0.679	0.862
Half-life (h)	0.90	0.07	0.80	1.02
C_0 (μg/ml)	34.40	3.72	30.37	42.45
AUC_{obs} (h*μg/ml)	42.82	8.71	32.46	55.69
$AUC_{0-\infty}$ (h*μg/ml)	42.83	8.71	32.46	55.71
$AUC_{\%extrap}$ (%)	0.02	0.01	0.00	0.04
V_z (ml/kg)	310.8	41.3	264.4	369.7
Cl (ml/kg/h)	242.3	48.9	179.5	308.0
$AUMC_{obs}$ (h^2*μg/ml)	60.59	22.77	32.03	97.00
$AUMC_{0-\infty}$ (h^2*μg/ml)	60.94	23.00	32.09	97.80
$AUMC_{\%extrap}$ (%)	0.52	0.44	0.03	1.40
MRT (h)	1.38	0.28	0.99	1.76
V_{ss} (ml/kg)	325.8	50.7	288.5	448.1

Abbreviations: λ_z, terminal rate constant; terminal half-life ($t_{1/2}$); $AUC_{\%extrap}$, percent of $AUC_{0-\infty}$ extrapolated; $AUC_{0-\infty}$, area under the curve extrapolated to infinity; AUC_{obs}, observed area under the curve; $AUMC_{\%extrap}$, percent of $AUMC_{0-\infty}$ extrapolated; $AUMC_{0-\infty}$, area under the moment curve extrapolated to infinity; $AUMC_{obs}$, observed area under the moment curve; C_0, concentration at time 0; Cl, clearance; MRT, mean residence time; V_{ss}, volume of distribution at steady state; V_z, volume of distribution by the area method.

in Figure 2. Although Cl also significantly changed with dosage ($p < .0001$), these changes were dosage specific. Cl did significantly decrease from the 5 mg/kg to the 20 mg/kg dosage ($p = .0011$). However, Cl at the 10 mg/mg dosage was significantly higher than 5 mg/kg ($p = .0388$) and 20 mg/kg ($p = .0004$) as shown in Figure 3. For most horses, AUC appeared to increase in a non-linear fashion (Figure 4).

3.2 | Adverse effects

There were no significant changes in physical examination parameters for any horse during the study. There was no evidence of swelling, heat or pain at the jugular catheter insertion sites and jugular vein refill remained normal for all horses throughout the study period.

TABLE 3 Non-compartmental pharmacokinetic parameters for thiamine hydrochloride in healthy horses (n = 9) after a single dosage of 20 mg/kg

Parameter	Mean	SD	Min	Max
λ_z (h^{-1})	0.632	0.094	0.482	0.726
Half-life (h)	1.12	0.18	0.95	1.44
C_0 (μg/ml)	69.38	7.61	59.53	82.68
AUC_{obs} (h*μg/ml)	123.64	26.85	96.42	171.28
$AUC_{0-\infty}$ (h*μg/ml)	123.68	26.86	96.43	171.32
$AUC_{\%extrap}$ (%)	0.03	0.04	0.00	0.11
V_z (mL/kg)	264.7	22.9	223.8	297.5
Cl (mL/kg/h)	167.9	32.4	116.7	207.4
$AUMC_{obs}$ (h^2*μg/ml)	244.97	95.73	139.15	404.09
$AUMC_{0-\infty}$ (h^2*μg/ml)	246.61	96.62	139.31	405.78
$AUMC_{\%extrap}$ (%)	0.60	0.68	0.11	2.25
MRT (h)	1.94	0.39	1.44	2.38
V_{ss} (ml/kg)	317.0	52.1	276.5	452.9

Abbreviations: λ_z, terminal rate constant; terminal half-life ($t_{1/2}$); $AUC_{\%extrap}$, percent of $AUC_{0-\infty}$ extrapolated; $AUC_{0-\infty}$, area under the curve extrapolated to infinity; AUC_{obs}, observed area under the curve; $AUMC_{\%extrap}$, percent of $AUMC_{0-\infty}$ extrapolated; $AUMC_{0-\infty}$, area under the moment curve extrapolated to infinity; $AUMC_{obs}$, observed area under the moment curve; C_0, concentration at time 0; Cl, clearance; MRT, mean residence time; V_{ss}, volume of distribution at steady state; V_z, volume of distribution by the area method.

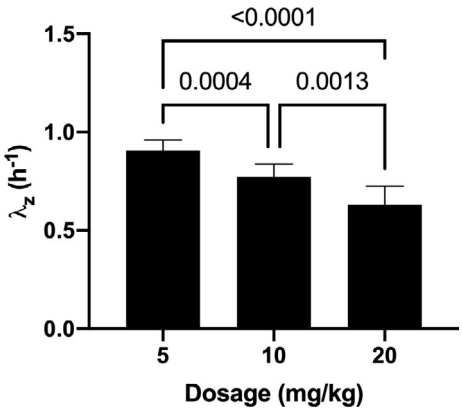

FIGURE 2 Terminal rate constant (λ_z) of thiamine hydrochloride after 5, 10, and 20 mg/kg IV dosing in healthy horses (n = 9)

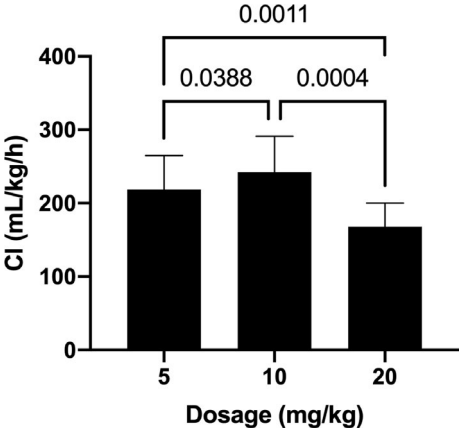

FIGURE 3 Clearance of thiamine hydrochloride after 5, 10, and 20 mg/kg IV dosing in healthy horses (n = 9)

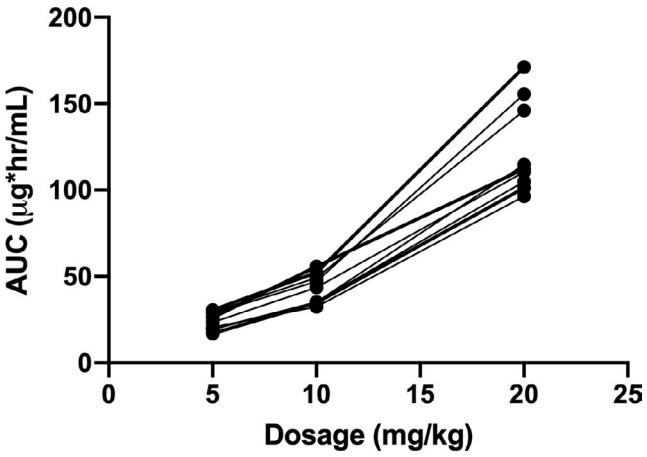

FIGURE 4 Area under the curve of thiamine hydrochloride after 5, 10, and 20 mg/kg IV dosing in healthy horses (n = 9)

4 | DISCUSSION

This is the first study to document the pharmacokinetics of IV TH administration in healthy horses or, to our knowledge, any large animal species. Despite common use of TH in small ruminants and cattle for treatment and prevention of polioencephalomalacia, pharmacokinetic studies have not been performed (Apley, 2015; Karapinar et al., 2008; Zhang et al., 2020). In our study, all three dosages administered resulted in supraphysiologic plasma thiamine concentrations with relatively short half-lives (0.77 ± 0.05–1.12 ± 0.18 h). We also documented that the terminal rate constant for thiamine significantly decreased with dosage. This, combined with non-linear increases in AUC with dosage, suggests that thiamine is eliminated in a non-linear, dosage-dependent fashion in horses.

Non-linear pharmacokinetics have also been reported for thiamine in humans. Weber et al. (1985) documented fourfold to10-fold differences in renal clearance between the initial and terminal phases of thiamine elimination (Weber & Kewitz, 1985). This phenomenon is attributed to handling by the kidney, which is the major route of thiamine excretion. As a small molecule, thiamine is freely filtered at the glomerulus and then either reabsorbed or secreted by tubular transporters in a concentration-dependent manner (Ott & Werneke, 2020). At low, physiologic concentrations, thiamine is reabsorbed by brush-border thiamine and organic cation transporters leading to minimal excretion and low renal clearance. Whereas, at high concentrations achieved by exogenous administration, thiamine is actively secreted, dramatically increasing renal clearance (Weber et al., 1990). Because thiamine secretion is transporter-mediated, it is also saturable which causes decreases in total plasma clearance at high concentrations. If these processes are similar in horses, they could partially explain the dosage-related differences in plasma clearance documented in the present study. The increase in Cl between 5 and 10 mg/kg could be due to a switch from renal reabsorption to secretion. However, in humans, this switch occurs around 200 nmol/L (0.053 μg/ml), which is well below plasma concentrations induced after IV administration of TH to horses. Thus, it is more likely that this difference in Cl is due to type II error, rather than renal transporter function. The lower thiamine Cl at 20 mg/kg compared to 5 and 10 mg/kg, however, could be evidence of transporter saturation.

Non-linear kinetics of thiamine in horses makes plasma concentrations difficult to predict for dosages outside of those studied (5, 10, and 20 mg/kg IV). Plasma concentrations during chronic therapy may also be difficult to predict, although thiamine's short half-life makes significant drug accumulation unlikely with intermittent dosing. However, before dosage and dosing frequency recommendations can be made, therapeutic targets must be established. Metabolic resuscitation protocols for sepsis in humans include a standard TH dosage of 200 mg (approximately 2.0–3.0 mg/kg) administered IV q 12 h (Donnino et al., 2016; Fujii et al., 2020; Marik et al., 2017). The rationale for this dosage is unclear but is centered around replacing thiamine that has been depleted during sepsis (Donnino et al., 2010).

No adverse reactions were observed after administration of IV TH in this study. Anecdotal reports of collapse and sudden death have been reported in small ruminants and cattle after IV administration, which has led to a general recommendation to dilute TH in 0.9% sodium chloride or sterile water and administer slowly (Apley, 2015; Cebra & Cebra, 2004). The horses in this study were administered IV TH over a 10 min period, so it is possible that more rapid administration might result in adverse effects.

As with most "first-in-species" pharmacokinetics reports, the small sample size of our study is a potential limitation. Inter-individual variation for many pharmacokinetic parameters was actually fairly low among our study participants; however, these values are unlikely to capture the full range of potential variability within the equine population. Similarly, our study design precluded assessing the effect of patient factors such as age, breed, disease status, or renal function on thiamine pharmacokinetics. Future population pharmacokinetic studies should be considered to address these factors, particularly since thiamine may have benefit in critically ill patients (Donnino et al., 2010; Marik et al., 2017). Another limitation is that we only quantified plasma-free thiamine in this study. Thiamine exists in several forms in the body including free thiamine, several phosphorylated forms, and thiamine bound to adenosine di- and triphosphate. Proportions of these moieties differ between plasma, red blood cells, and tissue with free thiamine and thiamine-monophosphate predominating in the plasma and the biologically active form, thiamine-diphosphate, predominating intracellularly (Ott & Werneke, 2020). Thus, quantification of the various forms of thiamine in both plasma and red blood cells would enhance understanding of thiamine distribution, metabolism, and potential for biologic effects in the horse. However, in humans, a single IV administration of TH increases plasma-free thiamine to a much greater degree than plasma thiamine-monophosphate or red blood cell thiamine-diphosphate so, for the purposes of this initial study, plasma-free thiamine quantification is likely sufficient (Tallaksen et al., 1993). Finally, endogenous thiamine from absorption from diet and hindgut microflora production (Carroll et al., 1949) may have interfered with determining the kinetics of exogenously administered thiamine. Because baseline (T0) concentrations and previously reported equine endogenous thiamine concentrations (Cymbaluk et al., 1978; Loew & Bettany, 1973) were so much lower than concentrations achieved after IV administration, this is unlikely to significantly affect results. Therefore, we chose to normalize the data by simply subtracting baseline concentrations.

In summary, a single IV TH injection in healthy horses achieved plasma thiamine concentrations well above endogenous levels and demonstrated a relatively short half-life. Thiamine hydrochloride appears to possess non-linear kinetics in the horse, likely due to complex renal elimination processes. Future pharmacodynamic studies are needed to identify therapeutic plasma concentrations of thiamine so rational dosage regimens can be established. Subsequent studies to investigate the effects of TH administration in septic horses can then be pursued.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

ANIMAL WELFARE AND ETHICS STATEMENT

This study was approved by the university's Institutional Animal Care and Use Committee (Protocol # 2003002025, Approval date: April 30, 2020).

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ORIGINAL ARTICLE

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A randomised, controlled trial to determine the effect of levothyroxine on Standardbred racehorses

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Abstract

Background: The use of thyroid supplement is pervasive in athletic horses although its effects on measures of performance are not known.

Objectives: One purpose of this study was to determine whether supra-physiologic doses of levothyroxine affect the velocities at which blood lactate was greater than 4 mmol/L (V_{La4}) and heart rate was over 150 (V_{150}) and 200 (V_{200}) beats per minute respectively. Additionally, a survey of post-race blood samples was also conducted to determine whether high thyroxine concentrations were common in racehorses.

Study design: A randomised, crossover, trial was performed in six healthy Standardbred racehorses.

Methods: Study 1: T4 was determined in 50 post-race samples from a single Standardbred meet. Study 2: Research horses were trained to fitness and then randomised to one of three treatments: carrier, 0.1 mg/kg thyroxine or 0.25 mg/kg thyroxine for 2 weeks. Horses completed a standardised exercise treadmill test (SET) to fatigue on the last day of treatment. Serum free and total thyroxine and triiodothyronine were determined on the day of SET testing. Blood lactate and ECG data were collected during the SET at 6, 8, 10, 11, and 12 m/s and during recovery. The effect of treatment and SET on heart rate and blood lactate was examined using generalised linear mixed models. Post hoc analysis was adjusted for multiple comparisons using Tukey's Test. Data were expressed as mean ± standard deviation and $P < .05$ was considered significant.

Results: Study 1: The median T4 value in this population of horses was 2.00 μg/dL (laboratory's normal range 1.5-4.5 μg/dL) and 3 of 50 racehorses had values above the laboratory reference range. Study 2: Levothyroxine at 0.25 mg/kg resulted in higher heart rates during SET (199 ± 30, 223 ± 17 and 239 ± 9 bpm at 6, 8 and 10 m/s respectively) and recovery (144 ± 20 and 119 ± 15 at 5 and 15 min) as compared to placebo (176 ± 18, 203 ± 10 and 219 ± 6 bpm at 6, 8, and10 m/s and 126 ± 5, 102 ± 11 at 5-15 minutes respectively). Three of six horses developed cardiac arrhythmias including atrial fibrillation.

Main limitations: A relatively small number of animals were used and a SET is not identical to actual racing conditions.

A portion of the data was presented as a poster at the 65th American College of Veterinary internal Medicine Forum, Phoenix, Arizona, USA 2019.

Conclusions: Supra-physiologic thyroxine supplementation caused a decreased V_{200} during a standard exercise test and may result in cardiac arrhythmias.

KEYWORDS
atrial fibrillation, horse, standardised exercise test, thyroid supplementation hyperthyroidism

1 | INTRODUCTION

The thyroid hormones thyroxine (T4) and triiodothyronine (T3) exert both direct and genomic effects that broadly act to set the body's metabolic rate. They mediate energy metabolism, oxygen consumption, and protein synthesis and degradation. Proper thyroid function is imperative for the success of athletes such as racing and performance horses.

Although documented, naturally occurring hypothyroidism is extremely rare in adult horses. Despite this, oral levothyroxine is often given to horses that suffer from a wide variety of nonspecific signs such as poor appetite, myositis, increased adiposity and lethargy. Thyroxine supplementation at a dose of 0.02 mg/kg once daily results in normal T4 and T3 concentrations in clinically healthy horses, while an increased dose of 0.04 mg/kg has been used to improve fat utilisation and induce weight loss in horses with equine metabolic syndrome.[1] Thyroxine and T3 are relatively stable molecules that can be accurately assayed from serum that has been stored frozen or refrigerated for several days. There is anecdotal evidence that some racehorses are given large amounts of thyroid supplement, perhaps in the belief that it improves performance. Additionally, it is not uncommon to detect blood T4 concentrations above the normal reference ranges in horses during post-race testing (B. Duncan, Ontario Racing Commission Veterinarian, personal communication). Despite the implication this finding has on equine welfare and racing outcomes, to date the effect of high doses of thyroid supplement on measures of performance in horses has not been reported.

There is a substantial body of literature on the effects of various forms of exercise on endogenous thyroid hormone concentrations and metabolism in many species including horses.[2-5] Physical exercise is associated with an increase in thyroxine turnover and rate of thyroid hormone de-iodination in both humans and horses.[6] It is believed that the increased turnover is due to increased uptake by muscle tissue. Despite the increased uptake, blood thyroid hormone concentrations are generally unchanged or decreased in conditioned humans, dogs and horses.[6-9] Many studies investigating the effect of exercise on blood thyroid concentrations sample athletes at rest rather than during or immediately following exercise. Blackmore and co-workers found that T4 was extremely low in racing Thoroughbreds, at times being beneath their level of detection.[7] Medica and co-workers reported that total and free T4 decreased in horses after a 6-week training program.[8] Both hyperthyroidism and hypothyroidism result in poor performance in human athletes. The cause is related to increased muscle weakness and poor cardiac function.[10-11] Hypothyroidism results in decreased athletic performance in horses referable to decreased cardiac function.[12,13]

There were two parts to this study. In the first, T4 concentrations were determined in a number of samples collected after racing. This was done to evaluate whether T4 concentrations outside the normal range are common in horses that have recently competed in vigorous exercise. The second arm of the study was conducted to investigate the effect of levothyroxine supplementation on the time to fatigue, treadmill velocity at which the heart rate reached 150 and 200 beats per minute, and blood lactate concentration reached 4 mmol/L in fit horses taking part in a standardised exercise test (SET). These measures, taken together, serve as a surrogate for performance because true race-time performance cannot be replicated under the controlled conditions of a high-speed treadmill study. The null hypothesis that we were testing was that administration of supra-physiologic doses of thyroxine have no effect on time to fatigue, the treadmill velocity at which heart rate was 150 and 200 beats per minute (V_{150} and V_{200} respectively), and treadmill velocity at which blood lactate concentration was 4 mmol/L (V_{La4}). Secondary outcomes were the serum concentrations of T4, free thyroxine (fT4) and T3 on the day of the SET, heart rate at the treadmill speeds as they were stepped up sequentially from 6 to 12 m/s, and the effect of serum thyroid concentrations on the primary outcomes of V_{150}, V_{200} and V_{La4}.

2 | MATERIALS AND METHODS

The research described was approved by the institutional animal care and use committee.

2.1 | Study 1

To gain a better appreciation of the typical range of serum T4 concentrations after exercise and to evaluate whether blood thyroid hormone concentrations outside the range determined in sedentary horses are a concern in racehorses, a convenience sampling of an anonymous group of 50 post-race blood samples was submitted for T4 determination. Samples were collected during a Standardbred meet at a local pari-mutuel racetrack. Samples were taken from horses that had been sent to the test barn because they either had won a race or were randomly selected by the race stewards for post-race screening. All blood was collected between 30 and 90 minutes after the horses completed their race. The samples that were analysed were the duplicate samples that were retained at the racetrack until the end of the meet. The blood was collected into serum separator tubes, spun when a clot had begun to form, between 30 minutes and 2 hours after collection,

and then frozen at −20°C. The tubes were de-identified before they were shared with the authors so there was no way to trace samples back to individual horses. Serum T4 was analysed by radioimmunoassay (RIA) at the Cornell University Animal Health Diagnostic Laboratory. The test was validated in the horse with a coefficient of variation of 5.2%.

2.2 | Study 2

2.2.1 | Study design

This was a randomised cross-over, masked, placebo-controlled study with six horses undergoing a SET after receiving three different dosages of levothyroxine supplement daily for 14 days (0, 0.1 mg/kg and 0.25 mg/kg). The specific details of the SET are given in Supplementary Item 1. Horses received each dosage of thyroxine for 2 weeks prior to a SET. There was a 2-week washout period between each dosing/SET period where horses received no supplement or carrier. Fitness was maintained during the washout period, however, by continuing a regular exercise programme. The washout period was determined to be of sufficient length in a previous study.[14] The individuals involved in the daily care, training and conducting the SET were blinded to the levothyroxine dosage.

Animals

Six Standardbred horses (4-9 years of age; 3 females, 3 geldings) were recruited for the study. A power calculation for sample size using the data in Ciloglu et al[2] between 45%-90% of maximum heart rate yielded a $P = .83$ with $\alpha = 0.05$ for a sample size of six. All the inputs used to determine the power calculation are given in Supplementary Item 2. Calculations were made using a commercially available statistical software package (ProcGLIMMIX SAS v.9.4o: SAS Institute).

Criteria for selection included the horse being in good overall health as determined by a normal physical examination, having a normal complete blood count, and no evidence of lameness or dynamic airway obstruction during treadmill evaluation at high speed (10 m/s). Horses were familiarised with laboratory surroundings, procedures, and acclimatised to running on the high-speed equine treadmill (EquiGym). All horses were conditioned 5 days per week for 6 weeks to establish uniform fitness prior to the start of the study as previously described.[15] Fitness level was assessed by determining V_{La4} every 2 weeks during training and appropriate fitness was considered achieved when V_{La4} reached a plateau.

Protocol

Treadmill tests were performed in a climate-controlled building (20-22°C). Horses underwent a SET designed to simulate race exertion.[15] A heart rate monitor (Polar Equine S-610: Polar Electro Inc,) and telemetric electrocardiogram system (Televet 100: Kruuse) were attached to the surcingle to continuously measure heart rate and ECG. A catheter (14 Ga × 13 cm Teflon catheter: Mila International, Inc,) was placed in the jugular vein using aseptic technique to allow blood collection during and after the SET. Fatigue was defined as the inability of the horse to maintain its position on the treadmill despite vigorous encouragement. The treadmill speed was slowed immediately at fatigue and the incline reduced to zero. Horses recovered by jogging 5 minutes at 5 m/s and then walking on the treadmill for an additional 10 minutes.

Blood lactate measurement

Blood was collected through the jugular catheter during the last 15 seconds of the 6, 8, 10 and 11 m/s SET speed steps and at the time of fatigue. In all instances, 10 mL blood was withdrawn from the catheter and discarded, after which 5 mL of blood was collected for analysis. The catheter was then flushed with 10 mL heparinised saline to maintain patency. Two additional lactate determinations were made on blood collected 5 and 15 minutes into the recovery period. Blood was collected in a 6 mL plastic syringe, injected into a 4-mL heparinised evacuated tube, and placed in crushed ice water immediately after collection. Blood L-lactate concentration was determined using a commercially available unit (Accutrend® Lactate unit: Roche Diagnostics Limited) previously validated in the horse[16] and a fixed volume of blood (25 μL). Lactate measurements were made within 5 minutes of sample collection. For each horse by dose combination, a unique regression upon SET data was performed to calculate V_{200} and V_{La4}, which was then analysed as a dependent variable in mixed models as described in the statistical methods. V_{La4} data were calculated by regression analysis using an exponential fit function in Excel. The relationship (La) vs speed during the incremental speed step fits an exponential function and this has been previously demonstrated.[17]

2.2.2 | Heart rate determination

The heart rate and ECG monitors were placed before the SET and data were collected during SET and for 15 minutes during the recovery period. Heart rate was recorded at 5 s intervals, analysed for rate using dedicated software (Polar Pro Trainer Equine Edition: Polar Electro) and stored in an ASCII file. The heart rate for each exercise speed was determined as the mean of the last 30 seconds for the interval. Screen captures of the ECG tracings were taken during the final 30 seconds of each step of the SET and at the 5- and 15-minute recovery times. They were evaluated for the presence of arrhythmias or other abnormalities by a board-certified cardiologist (MT) who was masked to horse, speed when ECG was captured, and thyroxine dosage. Other study personnel observed the ECG data in real-time while the SETs were taking place. For each SET, heart rate data were regressed against treadmill speed using linear regression with non-zero asymptote; with the resultant equation used to calculate values for V_{150} and V_{200}. For each individual test, heart rate was plotted against treadmill speed.[17]

Heart rate was also monitored during recovery. When arrhythmias were detected, the heart rate and ECG monitors remained in place for an additional hour or until normal sinus rhythm returned.

Thyroxine administration

Exogenous thyroid hormone was provided in the form of levothyroxine sodium powder formulated for horses (Thyro-L: Lloyd, Inc) and mixed with dextrose powder to assist with blinding. A pharmacist off site mixed Levothyroxine dosages or a control carrier vehicle (dextrose powder [Fischer Scientific]) into coded bags. The pharmacist retained the key to dose identification until the end of the study so that no person working with the horses on a daily basis was aware of the dose being given. Each morning the appropriate day's dosage was mixed into the morning grain meal. Horses were fed grain at a rate of 10% BW/day and observed to ensure that the entire dose was consumed. The order in which each horse received the three levels of levothyroxine (0, 0.1, and 0.25 mg/kg) was randomised using a random number generator (www.random.org). These dosages were selected as they produced serum T4 concentrations that were similar to those found in some horses with atrial fibrillation on post-race testing (B. Duncan, Ontario Racing Commission Veterinarian, personal communication). Serum T3 and T4 were assessed using a solid-phase competitive chemiluminescent immunoassay. Free T4 was determined using a 2-step process. Serum was dialysed against a buffer solution and then the T4 concentration in the dialysate was determined using RIA. All assays were previously validated in the horse with coefficient of variation of 6.5% (T4), 7.6% (T3) and 7.8% (fT4) respectively.[14]

Data analysis

Unless otherwise indicated, results are presented as mean ± standard deviation (SD). The effect of treatment on outcome variables as well as pre-, during- and post-test T3, total T4 and free T4 (equilibrium dialysis) was assessed using generalised linear mixed models using a commercial software package (ProcGLIMMIX SAS v.9.4o: SAS Institute). For each outcome, dose, the order in which doses were administered, and the study period were included as fixed effects and horse was included as a random effect to account for the correlation between repeated measurements. In the case of SET data, SET step and dose by SET step interactions were included as fixed effects, and in addition to the random effect of horse, a horse by dose interaction term was included to account for the repeated measures on each horse within each SET. Appropriate designation of data distributions as Gaussian was confirmed by visual evaluation of residual distributions. Significance of pair-wise comparisons was controlled by Tukey's post-hoc method. Level of significance was set at $P < .05$.

3 | RESULTS

3.1 | Study 1

The median T4 value in this population of horses was 2.00 μg/dL, which fell into the laboratory's normal range of 1.5-4.5 μg/dL (Figure 1). Three of the 50 samples had T4 concentrations higher than the laboratory's normal reference range, while 8 of the 50 were below the laboratory's normal reference range. Because the samples were de-identified, it was not possible to correlate thyroid hormone concentration to any measure of performance.

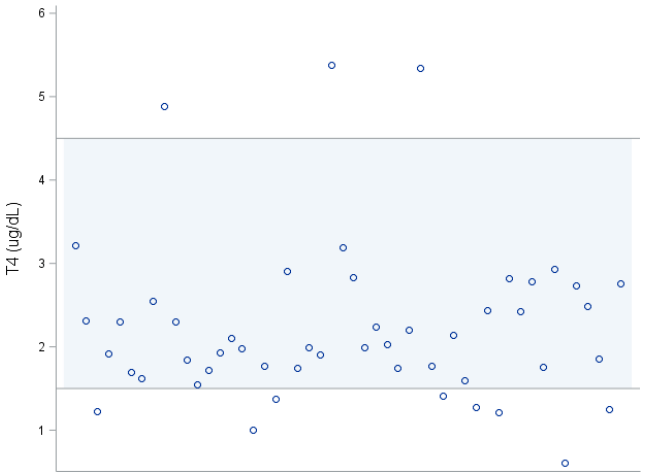

FIGURE 1 Total T4 concentrations in post-race serum samples from 50 Standardbred racehorses. Normal reference range is 1.5-4.5 μg/dL. Each point along the X-axis represents an individual horse

3.2 | Study 2

Overall, the horses tolerated the training schedule and thyroxine administration well. Serum concentrations of T4, free T4 as measured by equilibrium dialysis, and T3 increased in a dose-dependent manner when supra-physiologic dosages were given (Table 1). The concentrations of T4 and fT4 when the horses were on both the 0.1 and the 0.25 mg/kg dosages of levothyroxine were higher from those when horses were not receiving supplement. Serum T3 concentrations when horses received 0.25 mg/kg were increased over those not receiving supplement, but at the 0.1 mg/kg dosage they were not.

3.2.1 | Assessment of SET

Run time to fatigue was not different between the three treatment groups. There was no difference in lactate concentrations between treatment groups at any time point. The V_{La4} for the 0, 0.1 and 0.25 mg/kg portions of the study were 9.20 ± 0.80, 9.23 ± 0.88 and 9.31 ± 1.04 respectively. There was no difference between treatment groups or between the three dosages used in the study (Figure 2).

The 95% Confidence interval and the model-calculated mean heart rate were over 150 at 6 m/s and above, thus V_{150} could not be evaluated. The effect of levothyroxine administration on V_{200} in metres per second were 7.82 ± 138, 7.6 ± 1.01 and 5.97 ± 1.89 at the 0, 0.1 mg/kg and 0.25 mg/kg dosages respectively. The high dosage value of 5.97 ± 1.89 was significantly different from control (Tukey adjusted $P = .02$) and 0.1 mg/kg dose (Tukey adjusted $P = .04$) values.

TABLE 1 Effect of levothyroxine administration on mean ± SD blood concentrations of T4, fT4(dialysis) and T3. Samples collected after 14 days of levothyroxine administration

Dose	0	0.1 mg/kg	0.25 mg/kg	Normal Range
T4 (μg/dL)	1.17 ± 0.40^{A}	4.41 ± 2.36^{B}	7.78 ± 3.36^{B}	1.0-3.0
T3 (ng/dL)	84 ± 22^{A}	93 ± 34^{A}	136 ± 32^{B}	30-80
fT4 (ng/dL)	1.38 ± 0.32^{A}	3.37 ± 2.10^{B}	5.81 ± 2.30^{B}	1.2-1.8

Note: Different letters denote statistically significant differences (Tukey test adjusted $P < .05$). Figures designated A are statistically different from figures designated B.

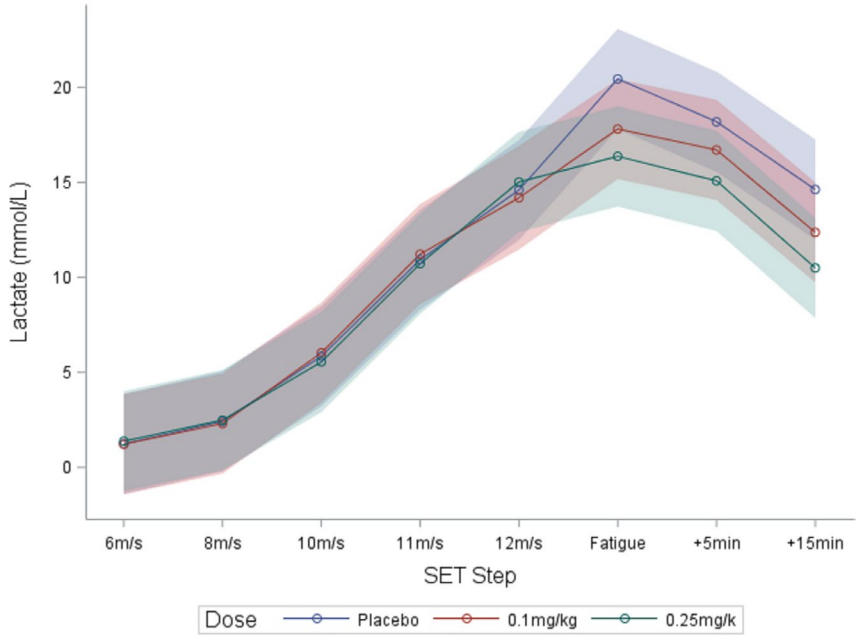

FIGURE 2 Effect of three levothyroxine doses on blood lactate concentration during a standardised exercise test and subsequent recovery phase. Lactate concentration with 95% confidence limits given

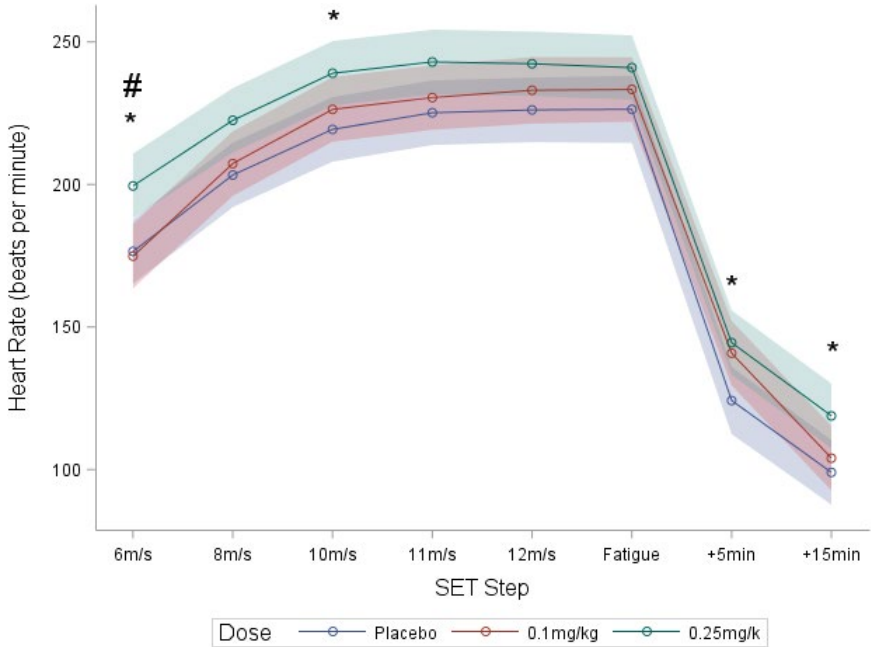

FIGURE 3 Effect of three levothyroxine doses on heart rate during a standardised exercise test and subsequent recovery phase. Heart rates with 95% confidence limits given. # = High dose is significantly higher than low dose (Tukey's Test adjusted *P*-value < .05). * = High dose is significantly higher than control (Tukey's Test adjusted *P*-value < .05)

3.2.2 | Heart rate

In Figure 3, the heart rate for each dose is plotted against the SET step, showing the model calculated mean and 95% confidence interval for each dose of thyroxine at each step of the SET. Levothyroxine at 0.25 mg/kg resulted in higher heart rates during SET (199 ± 30, 223 ± 17 and 239 ± 9 bpm at 6, 8 and10 m/s respectively) and recovery (144 ± 20 and 119 ± 15 at 5 and15 min) as compared to placebo

(176 ± 18, 203 ± 10 and 219 ± 6 bpm at 6, 8 and10 m/s and 126 ± 5, 102 ± 11 at 5-15 minutes respectively).

Resting T3 concentrations affected heart rate ($P = .015$), but there was no interaction between T3 and time upon heart rate ($P = .97$) during the SET. For a given time point, an increase of ten units in T3 concentration was associated with an increase in heart rate by 2.3 beats per minute. Resting T4 concentrations also affected heart rate ($P = .0007$). For a given time point, an increase of one unit in T4 concentration was associated with an increase in heart rate by 2.7 bpm. There was no interaction between resting T4 and time upon heart rate during the SET ($P = .6$). Resting free T4 concentrations affected heart rate ($P = .002$) but there was no interaction between resting free T4 and time ($P = .6$) upon HR during the SET. For a given time point during the SET, an increase of one unit in free T4 concentration was associated with an increase in heart rate by 3.8 bpm.

Levothyroxine treatment was associated with arrhythmias in three of the six horses, in all instances when the horse was receiving the highest dose of thyroxine. In two instances premature atrial depolarisations were noted. They were not present before the SET commenced and resolved within 40 minutes after the SET was concluded. In a third instance, a horse on the high dose of thyroxine developed atrial fibrillation during the SET. The arrhythmia persisted for several hours although the horse spontaneously reverted to normal sinus rhythm within 24 hours. Since SETs were conducted on the final day of thyroid hormone supplementation for each arm of the study, the affected horse was not given any thyroxine the following morning. Thus, there is no way to determine if the arrhythmia would have persisted if thyroxine administration had been continued. The horse experienced no further arrhythmias.

Serum T3 concentration had no effect on V_{200} ($P = .09$), but T4 and free T4 were associated with a more rapid time to V_{200}. For each unit increase in total T4 concentration, V_{200} decreased by 0.21 m/s ($P = .04$). For each unit increase in free T4 concentrations, V_{200} decreased by 0.30 m/s ($P = .04$).

There was a difference in V_{La4} between the six possible sequences of drug administration ($P < .001$). Since each horse had a unique sequence of levothyroxine administration, this is likely due to individual horse variation and the cumulative effect of prolonged training. For each unit increase in T3 concentrations, V_{La4} calculated by exponential interpolation increased by 0.0048 ($P = .03$). There were no effects of resting T4 or free T4 concentration on V_{La4}.

There was no difference between thyroxine dose and time to fatigue. Similarly, there was no effect of T3 ($P = .9$), T4 ($P = .8$), or free T4 ($P = .9$) concentrations on time to fatigue.

4 | DISCUSSION

4.1 | Study 1

In all, 11 of the 50 horses whose post-race samples were tested had thyroxine values outside the reference range. Because 39 of the samples were within the range that was established in sedentary horses, it did not appear that major alterations in serum T4 concentrations occur in horses after vigorous exercise. The lower than normal values are consistent with the observation that fit horses often have lower T4 concentrations than sedentary horses.[7,8] Because the samples were de-identified, there was no way to compare T4 values to racing outcomes or race times.

4.2 | Study 2

The horses that took part in the crossover study generally tolerated the training schedule. Naturally occurring hyperthyroidism is rare in horses. When it has been described it has been associated with functional thyroid tumours in older animals.[18,19] Clinical signs include weight loss, tachycardia, hyper excitability, and sweating. Frank and co-workers demonstrated that giving twice the replacement dose of thyroxine to healthy adult mares produced weight loss and improved glucose utilisation.[20] No exercise parameters were measured in that trial, although they did document normal cardiac function and no change in resting heart rate.

The dosages of 0.1 and 0.25 mg/kg are 2 and 5 times the recommended amounts of levothyroxine supplementation needed to achieve normal serum concentrations.[21] They were selected in this experiment because they resulted in blood T4 concentrations that were similar to those found in post-race samples of horses with suspected or confirmed atrial fibrillation (B. Duncan, Ontario Racing Commission Veterinarian, personal communication). Atrial fibrillation is the most common pathological arrhythmia in horses and is common in Standardbred racehorses.[22] In humans, thyrotoxicosis is a reason for the development of atrial fibrillation without identified underlying cardiac disease.[23] The presence of arrhythmias in the horses during this study may be due to the alterations in calcium channel function and subsequent contractility that occurs in the presence of increased thyroid hormone concentrations.[24]

There was no difference in lactate concentrations and V_{La4} between treatment groups or study arms, indicating that the horses maintained the same level of fitness throughout the trial. All blood was collected from the jugular vein. While constituent concentrations in jugular blood may not be identical to those found in blood collected from other sites or mixed venous blood, the use of jugular samples is consistent with other studies in exercising horses. For the purpose of our study, small differences between jugular and mixed venous were not pertinent as it was used to document fitness and then compare fitness level between the three dosages used in the study.

The running speed corresponding to a heart rate of 200 beats per minute occurred earlier (lower V_{200}) when horses were on the high dose thyroxine treatment (0.25 mg/kg, $P < .05$). This more rapid rise in heart rate for a given workload would be expected to translate in decreased performance since V_{200} normally increases in parallel with performance and higher values indicate superior aerobic capacity.[25]

The use of thyroid hormone supplement is not regulated by most racing and performance horse jurisdictions, so there is no way to determine how much supplementation is occurring. In addition to affecting cardiac and skeletal muscles, iatrogenic hyperthyroidism could potentially have negative effects on other body systems. Cardiorespiratory function and oxygen exchange are decreased when hyperthyroidism is present.[26] In humans, both subclinical and clinical hyperthyroidism are responsible for decreased bone mineral density[27] and increased risk of fracture.[28,29] Thyroid dysfunction has also been linked to tendon injury in humans.[30] Musculoskeletal breakdown is a major problem in horse racing, and ensuring that horses do not compete if they have elevated blood concentrations of thyroid hormones could remove hyperthyroidism as a potential contributing factor to catastrophic injury. Certainly, much more research on the effects of iatrogenic levothyroxine administration on musculoskeletal health is needed.

Limitations of this study include the relatively small sample size of six horses. The size limitation was mitigated by the fact that each horse served as its own control. Additionally, a SET is not the same as an actual race or athletic endeavour.

Levothyroxine treatment was associated with arrhythmias in three of the six horses studied including atrial fibrillation. The impact of exogenous levothyroxine administration on factors that influence equine athletic performance merits further study, and the welfare implications of its administration are numerous and must be considered by regulatory bodies.

CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

J. Kritchevsky, S. Tinkler, L. Couetil and L. Forsythe contributed to the study design. J. Kritchevsky, S. Tinkler, L. Couetil, L. Forsythe, C. Olave, M. Tropf and K. Ivester contributed to study execution. J. Kritchevsky, L. Couetil, M. Tropf and K. Ivester contributed to the data analysis and interpretation. J. Kritchevsky prepared the manuscript with contributions from L. Couetil, S. Tinkler and K. Ivester. All authors approved the final version of the manuscript.

INFORMED CONSENT

Not applicable.

ETHICAL ANIMAL RESEARCH

The described research was approved by the Purdue University Animal Care and Use Committee (Approval number: 1703001548).

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13480.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Dust exposure and pulmonary inflammation in Standardbred racehorses fed dry hay or haylage: A pilot study

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ABSTRACT

Respirable dust exposure is linked to airway inflammation in racehorses. Feeding haylage may reduce dust exposure by 60–70%. The objective of this study was to compare dust exposure, airway cytology, and inflammatory cytokine concentrations between horses fed haylage or hay over 6 weeks while in training. Seven healthy Standardbred horses were randomly assigned to be fed alfalfa hay (n = 3) or grass-alfalfa mix haylage (n = 4) for six weeks while training on a treadmill. Dust exposure was measured gravimetrically at the breathing zone. Endotoxin and β-glucan concentrations in respirable dust were measured. Bronchoalveolar lavage fluid (BALF) cytology was determined at baseline and after 2, 4, and 6 weeks. Cytokine concentrations (interferon-γ, tumor necrosis factor-α, and interleukin-4) were measured in BALF at baseline and week 6. The effect of forage on exposure, airway cytology and cytokines were evaluated using generalized linear mixed models.

Respirable dust and β-glucan exposures were lower in horses fed haylage than hay (0.02 ± 0.001 mg/m^3 vs. 0.06 ± 0.01 mg/m^3; P = 0.03, and 69 ± 18 pg/m^3 vs. 160 ± 21 pg/m^3; P = 0.02, respectively). In horses eating haylage, BALF neutrophil proportion decreased between baseline (2.2 ± 0.5%), week 2 (0.8 ± 0.3%; P = 0.01) and week 6 (0.7 ± 0.2%; P = 0.03). By week 6, horses fed haylage had lower BALF neutrophilia than horses fed hay (4.0 ± 0.7 %; P = 0.0004). Interleukin-4 concentration in BALF was higher at week 6 (14.4 ± 4.6 pg/mL) in horses fed hay compared to baseline (2.9 ± 4.6 pg/mL; P = 0.007). In conclusion, feeding haylage instead of hay to horses in training can reduce exposure to respirable irritants and mitigate airway neutrophilia.

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Introduction

Mild-moderate equine asthma (EA), previously known as inflammatory airway disease, is a non-septic inflammatory disease of the equine respiratory system (Couëtil et al., 2016). Clinical signs may include decreased performance and chronic, intermittent cough (Holcombe et al., 2006; Bedenice et al., 2008). Diagnosis is confirmed by detecting excessive tracheal mucus with endoscopy or increased proportion of inflammatory cells on bronchoalveolar lavage fluid (BALF) cytology (Couëtil et al., 2016). In Ivester et al. (2018) 80% of actively racing Thoroughbreds exhibited airway inflammation based on BALF cytology.

Horses are exposed to high concentrations of aerosolized particles inside barns (Ivester et al., 2014a). Exposure to barn environment is associated with airway disease both in horses (Tesarowski et al., 1996; Ivester et al., 2018) and people working in horse barns (Mazan et al., 2009). Barn dust contains a variety of organic particles such as fungi, molds, endotoxin, β-glucan, debris, and bacteria (Clements and Pirie, 2007; Ivester et al., 2014a). Two major pro-inflammatory components of organic dust are β-glucans, originating mainly from fungi and plant cell walls, and bacterial endotoxins (Douwes et al., 2003). The main source of respirable dust in stabled horses is dry hay (Ivester et al., 2014b). Feeding hay is associated with up to a 10-fold increase in exposure to airborne dust in the horses' breathing zone when compared to pasture (McGorum et al., 1998). Other forage options with lower dust exposure are available. Haylage, for example, results in a 60–70 % reduction in breathing zone measures of dust exposure compared to hay (Clements and Pirie, 2007). Haylage is typically grown and cut at similar stages as hay, except that it is harvested when the moisture is still high (30–50%) (Finch et al., 2014). Silage is also harvested and wrapped when moisture is high (>50%). Feeding silage to severely asthmatic horses during stabling can maintain them in clinical remission (Vandenput et al., 1998), but whether feeding haylage to racehorses in training benefits airway health is currently unknown.

Experimental exposure of healthy horses to dusty hay results in neutrophilic airway inflammation (Robinson et al., 2003). In

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natural conditions, neutrophilic airway inflammation in racing Thoroughbreds is correlated with respirable dust and endotoxin exposure while mast cell inflammation is associated with β-glucan exposure (Ivester et al., 2018). Both types of inflammation are negatively related to performance (Ivester et al., 2018). Feeding horses with severe EA a low dust diet helps reduce airway inflammation and improves clinical signs such as performance and cough (Nogradi et al., 2015). However, it is unknown if reducing exposure to respirable dust and associated irritants in healthy horses in training decreases airway inflammation.

Cytokine profiles associated with cytological phenotypes of mild-moderate EA are controversial (Lavoie et al., 2011; Beekman et al., 2012; Richard et al., 2014). Neutrophilic inflammation in horses may result from an activation of the innate immune system with an increase in tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) concentration in BALF (Richard et al., 2014) but others reported only increased expression of interleukin (IL)-1β (Lavoie et al., 2011). An increase in helper T-cell type 2 (Th-2) cytokine expression, such as IL-4 in BALF, has also been reported in horses with mast cell inflammation (Lavoie et al., 2011; Beekman et al., 2012).

Therefore, the objective of this study was compare dust exposure, airway cytology and BALF cytokines between horses fed haylage or hay over 6 weeks while in training. We hypothesized that horses in training fed haylage for 6 weeks will be exposed to lower respirable irritants (dust, endotoxin, β-glucan) and exhibit significantly lower BALF neutrophil and mast cell proportions, and pro-inflammatory cytokine concentrations (IL-4, TNF-α, IFN-γ) than horses fed dry hay.

Materials and methods

Experimental design

A prospective clinical trial was designed as a pilot study to compare the effect of forage on dust exposure and airway inflammation in horses during training. Seven healthy Standardbred racehorses (3 mares and 4 geldings) between 4 and 9 years old and bodyweight 454 ± 45 kg were used in this study. Prior to enrollment, horses had been housed at a single private facility on pasture during the day and stabled during the night. For the study, horses were housed in the same climate-controlled barn sharing air space in individual stalls bedded with wood shavings and managed identically with the exception of the assigned forage. Horses were trained using a protocol similar to a previous study (Perez-Moreno et al., 2009). Briefly, they were trained 5 days a week on a high-speed treadmill over a 6 week period to establish uniform fitness

After enrollment, horses were allocated into two groups, one fed good quality alfalfa hay (n = 3) and the other fed grass-alfalfa mix haylage (n = 4) using simple randomization through a random number table. Both forages were fed on the ground. Feed consumption was measured daily and horses weighed weekly. Those horses assigned to be fed grass-alfalfa mix haylage were gradually transitioned from good quality alfalfa hay to grass-alfalfa mix haylage over a period of 7 days. Consumption of forages increased progressively from 1.8% of body weight as fed per day during week 1–2.2% of body weight as fed per day by week 6. Horses were also fed increasing amounts of concentrate (Omolene 200, Purina Animal Nutrition) in accordance to the training intensity and consumption increased from 0.2% body weight/day at the start of training up to 1% body weight by the end of week 6. At baseline, physical examination, hematology, and bronchoalveolar lavage (BAL) were performed on each horse. Horses were enrolled if they were healthy based on physical examination and complete blood count. Horses were monitored daily by recording rectal temperature, respiratory and pulse rates and feed consumption. Physical examination and BAL were repeated at weeks 2, 4 and 6. Dust exposure was measured at the breathing zone of each horse on two occasions between week 4 and week 6. Horses achieved peak fitness by the end of week 6 as indicated by the plateau reached by the horses' speed for a blood lactate of 4 mmol/L. The Purdue University Animal Care and Use Committee approved all procedures (Protocol number 1703001548A004; Approval date, 4 March, 2017).

Breathing zone respirable particulate measurements

Respirable and inhalable particulate samples were collected in the horse's breathing zone over the course of 6 h using a personal sampler secured to the noseband of the halter as previously described (Ivester et al., 2012). The respirable fraction (particles with 50% cutoff of 4 μm) was collected onto 37 mm glass fiber filters using an aluminum cyclone (P225-01-02, SKC, Inc.) and the inhalable fraction (particles with 50% cutoff of 100 μm) onto 25 mm PVC filters using an Institute of Medicine sampler (IOM, SKC). Both samplers were connected by flexible tubing (Tygon) to sampling pumps (AirChek 2000, SKC) which were secured to a surcingle on the girth of the horse. The horse was free to eat, drink, and move around the stall as usual. The change in weight of each filter was divided by the volume of air sampled to determine dust exposure in mg/m^3.

Beta-glucan and endotoxin analysis

Respirable dust samples were stored at −20 °C until measurement of β-glucan and endotoxin. The content of β-glucan and endotoxin in the respirable dust was measured using a kinetic chromogenic Limulus amebocyte lysate (LAL) technique (NexGen PTS0, Charles River Laboratories) as previously described (Ivester et al., 2018).

Bronchoalveolar lavage

Horses were sedated with butorphanol (0.02–0.04 mg/kg IV; Torbugesic, Zoetis) and with xylazine hydrochloride (0.2–0.5 mg/kg IV; AnaSed, Akorn Animal Health). A sterile BALF tube (300 cm long; 10 mm outer diameter; Bivona Medical Technologies) was passed through the nose and wedged into a distal bronchus. Two hundred fifty mL of sterile 0.9% sodium chloride were instilled and recovered using 60 mL syringes as a single aliquot. BALF was filtered through sterile gauze and immediately placed on ice and processed within 1 h of collection. Cytospin preparations were performed using 200 μl of BALF and slides processed with modified Wright stain. Differential cell count was determined on 600 cells by a single individual (CO) using a 5-field technique by counting a maximum of 120 cells in each of at least 5 fields (40X oil immersion) until a total of 600 leukocytes was reached (Fernandez et al., 2013). Epithelial cells were not included in the cells counted. The individual reading slides was masked to horse identity and time point.

Cytokine measurements

ELISA tests were performed on BALF supernatant from baseline and week 6. All BALF samples were kept on ice until centrifugation (100 × g for 10 min) within 30 min of collection and supernatant samples were stored at −80 °C until analyses. BALF samples were diluted as needed. TNF-α, IL-4 and IFN-γ were measured using equine-specific ELISA kits (R and D Systems) according to the manufacturer's instructions as previously described (Richard et al., 2014). Measures were performed in duplicate and average recorded.

Data analysis

Repeated measures generalized linear models were constructed to determine the effect of forage on dust exposure, BALF volume recovery, cytology, and cytokine concentrations (Ivester et al., 2018). Residual plots were visually evaluated to check model assumptions as well as correct specifications of distribution and link functions. Significance of post hoc pairwise comparisons was controlled by Tukey's post hoc method, and an adjusted P value of <0.05 was considered significant. Data analyses were performed using ProcGLIMMIX SAS v.9.4 and graphs were made with GraphPad Prism 8.

Results

None of the horses developed any signs of respiratory disease. Both feeding protocols were well tolerated, and no adverse effects were observed during the duration of the study.

Exposure to organic dust

Fourteen exposure assessments were performed, two for each horse, between weeks 4 and 6. No measure of exposure varied between the first and the second exposure assessment ($P > 0.2$). Respirable dust exposure was lower in the horses eating haylage when compared with horses eating hay (P = 0.029; Fig. 1). Exposure to inhalable dust was not different between groups (P = 0.25; Fig. 2). Respirable β-glucan exposure was also lower in horses eating haylage when compared with horses eating hay (P = 0.025; Fig. 3). Respirable endotoxin concentration was not different between groups (Hay, 1.62 ± 0.84 EU/m^3; Haylage, 1.74 ± 0.86 EU/m^3; P = 0.79).

Airway cytology

The mean BALF recovered was 190 ± 17 mL corresponding to 76.0 ± 6.8 % of the volume instilled. BALF recovery volume did not

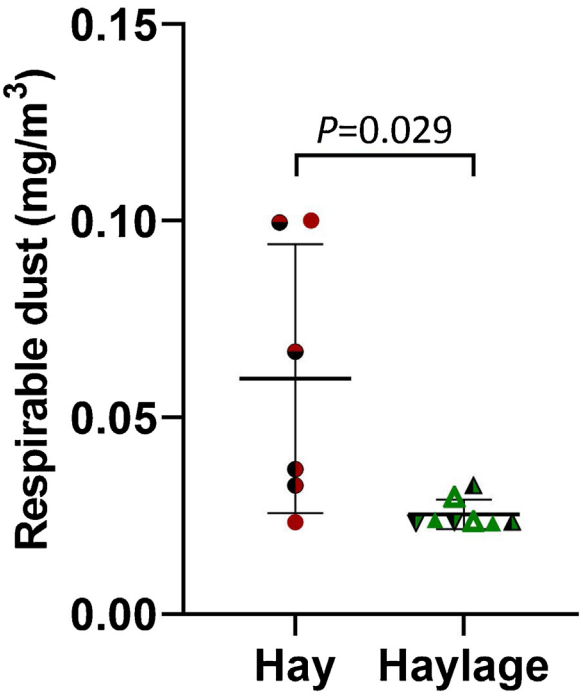

Fig. 1. Mean and standard deviation of respirable dust exposure in the breathing zone of horses fed hay or haylage. Hay $n = 6$, haylage $n = 8$ (two measurements per horse). Identical symbols represent paired data points.

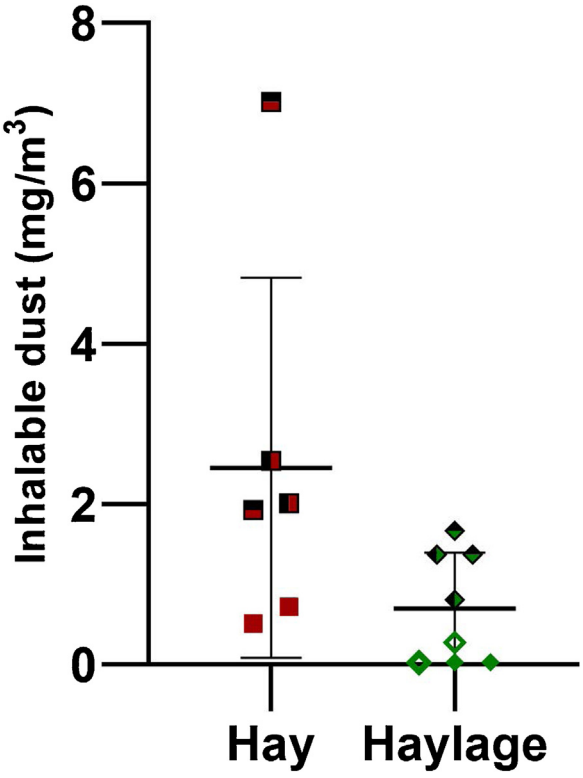

Fig. 2. Mean and standard deviation of inhalable dust exposure in the breathing zone of horses fed hay or haylage. See Fig. 1 for keys.

vary between groups ($P = 0.96$), over time ($P = 0.26$), and there was no difference between groups over time ($P = 0.22$). At baseline three horses (one in the haylage group and two in the hay group) demonstrated BALF mast cell inflammation (>2%), but all had

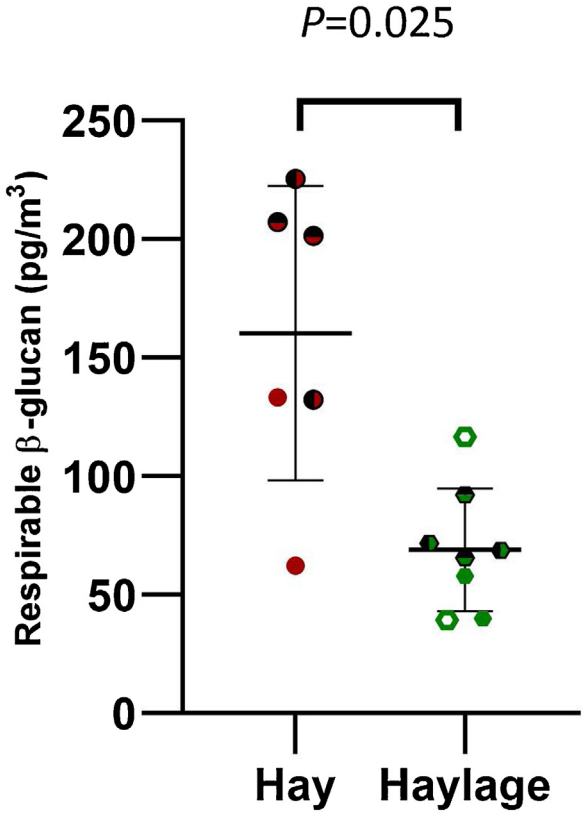

Fig. 3. Mean and standard deviation of respirable β-glucan exposure in the breathing zone of horses fed hay or haylage. See Fig. 1 for keys.

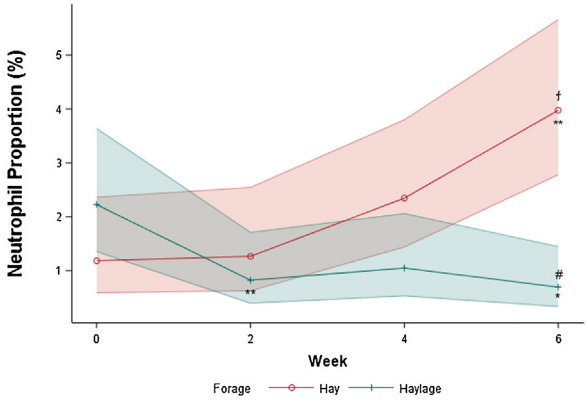

Fig. 4. Bronchoalveolar lavage fluid (BALF) neutrophil proportion in horses fed hay or haylage for six weeks. Lines represent the mean and bands represent 95% confidence interval. * Denotes differences compared to baseline * $P = 0.03$ ** $P = 0.01$. † Denotes differences with week 2 $P = 0.001$. # Denotes differences between groups $P = 0.0004$.

normal neutrophil proportions (<5%). BALF neutrophil proportions were affected by forage assignment over time ($P = 0.002$). The proportion of neutrophils was lower by week 2 in horses eating haylage when compared with baseline ($P = 0.01$; Fig. 4). By week 6, horses fed haylage still had a lower proportion of BALF neutrophils compared to baseline ($P = 0.03$) and compared to horses fed hay at week 6 ($P = 0.0004$). The proportion of neutrophils in the horses fed hay was higher at week 6 when compared with baseline and week 2 ($P = 0.01$ and $P = 0.001$, respectively). Other BALF cell proportions were not different between horses fed hay or haylage at any time point or within group over time (Table 1).

Table 1
Summary of bronchoalveolar lavage fluid (BALF) cell proportions from seven healthy Standardbred racehorses fed hay (n = 3) or haylage (n = 4) for 6 weeks during training. Data presented as mean ± standard deviation.

	Time	Hay	Haylage
BALF neutrophil proportions (%)	Baseline	1.2 ± 0.7	2.2 ± 1.0
	Week 2	1.3 ± 0.7	0.8 ± 0.4
	Week 4	2.3 ± 0.9	1 ± 0.7
	Week 6	4.0 ± 1.2	0.7 ± 0.5
BALF mast cell proportions (%)	Baseline	2.7 ± 1.3	1.7 ± 1.1
	Week 2	3.5 ± 1.5	1.7 ± 1.0
	Week 4	3.0 ± 1.3	2.1 ± 1.2
	Week 6	2.4 ± 1.1	2.3 ± 1.1
BALF eosinophil proportions (%)	Baseline	0.3 ± 0.4	0.1 ± 0.2
	Week 2	0 ± 0	0 ± 0
	Week 4	0.1 ± 0.3	0.1 ± 0.3
	Week 6	0.3 ± 0.4	0.1 ± 0.2
BALF macrophage proportions (%)	Baseline	57.0 ± 7.5	58.9 ± 3.6
	Week 2	60.6 ± 7.4	67.8 ± 5.2
	Week 4	56.6 ± 5.8	63.8 ± 0.8
	Week 6	53.1 ± 2.1	64.9 ± 4.6
BALF lymphocyte proportions (%)	Baseline	39.0 ± 7.8	37.3 ± 2.9
	Week 2	34.9 ± 7.0	29.9 ± 5.2
	Week 4	38.0 ± 5.4	33.1 ± 1.7
	Week 6	40.2 ± 2.2	32.3 ± 4.3

Cytokine concentrations

The BALF concentration of IFN-γ, and TNF-α did not vary between horses eating hay or haylage over time (P = 0.8 and P = 0.7, respectively; Fig. 5). The BALF IL-4 concentration differed between the groups over time (P = 0.008), due to a significant increase in IL-4 concentrations measured after 6 weeks in horses eating hay (P = 0.007; Fig. 5) when compared to baseline while no change was detected in horses fed haylage.

Discussion

Feeding haylage to horses during training resulted in a lower exposure to respirable dust and β-glucan concentrations when compared to horses fed hay but did not affect exposure to inhalable dust or respirable endotoxin. Pulmonary neutrophilia was reduced as early as 2 weeks after feeding haylage, while horses eating hay demonstrated increased BALF neutrophil proportions between weeks 2–6. BALF IL-4 concentrations increased over the course of 6 weeks in horses fed hay but did not change in those fed haylage. Results from this pilot study suggest that feeding low dust forage such as haylage without any other management changes is sufficient to improve airway health in racehorses in training.

Haylage is a conserved grass that is considered a cross between hay and silage (Manley et al., 2019). This forage is cut and allowed to dry but it is wrapped in airtight plastic bags when the moisture is about 65% (Finch et al., 2014). One of the main concerns about feeding haylage is the potential risk of *Clostridium botulinum* toxin ingestion if the forage is not preserved properly (e.g. puncture of plastic cover). To minimize these risks, it is recommended to carefully examine the bales for the presence of molds, and for bales to be fed within a 3–7 days of opening (Geor, 2008; McGorum et al., 2013). A killed vaccine against *C. botulinum* type B is available and should be considered. However, botulism may be caused by other serotypes and there is no cross-protection between serotypes. None of the horses in this study were vaccinated and no side effects developed while horses were eating haylage. However, care was taken to discard any part of the bale that appeared grossly molded before feeding the horses.

Deposition of airborne particles in airways is dependent on breathing pattern and particle aerodynamic diameter. In humans, inhalable dust is defined as particles with an aerodynamic diameter of 100 μm at which the likelihood of a particle this size entering the respiratory tract is 50% (Ivester et al., 2014a). Similarly, respirable particles are defined as particles with an aerodynamic diameter of 4 μm at which the likelihood of a particle this size entering the bronchio-alveolar region is 50%. Various air samplers are available to measure exposure to inhalable and respirable dust in humans however, it is unclear which size fraction is most appropriate to assess the effect of dust exposure on horse's airway health. Therefore, both inhalable and respirable dust fractions were measured in this study.

Exposure to respirable dust was three times lower in horses eating haylage when compared to hay, despite the fact that horses were housed in the same air space with the same bedding. The exposures measured in this study were similar to a previous study reporting mean breathing zone respirable dust exposures of 0.064 ± 0.04 mg/m^3 and 0.026 ± 0.01 mg/m^3 when a pony was housed on shavings and fed either hay or haylage, respectively (Clements and Pirie, 2007). Respirable dust breathing zone exposures in the horses eating hay were also similar to those reported in Thoroughbred racehorses fed dry hay and bedded on sawdust (0.055 ± 0.09 mg/m^3; Ivester et al., 2014b, 2018). In contrast, breathing zone measures of respirable dust exposure reported in the current study are markedly lower than those reported for a single pony housed under the low dust conditions of shavings and silage (0.22 mg/m^3) and instead comparable to the measures obtained when the same horse was at pasture (0.08 mg/m^3; McGorum et al., 1998). Dust exposure is also affected by individual horse eating behavior with some horses burying their noses while eating hay or shaking it, therefore, resulting in higher exposure in the breathing zone (Ivester et al., 2014b). The eating behavior of the pony used in their study may have contributed to the higher dust exposure (McGorum et al., 1998). Additionally, differences in sampling techniques and equipment or quality of the forage may have contributed to the variability. Furthermore, those studies did not report the effect of varying exposure levels on airway cytology making management recommendations difficult to issue. Exposure to inhalable dust was not different between groups and did not appear to impact BALF neutrophil proportions. These findings are in agreement with previous studies that found no association between exposure to inhalable dust in the breathing zone and airway inflammation in racehorses (Ivester et al., 2014b; Ivester et al., 2018).

Respirable β-glucan exposure was lower with haylage compared to hay. Horses eating hay were exposed to almost 3 times higher β-glucan levels (160 ± 21.3 pg/m^3) than in a previous study of racehorses eating hay while bedded on sawdust bedding (55.5 ± 66.2 pg/m^3), similar to the exposure of horses eating haylage (69 ± 18.4 pg/m^3) in the present study (Ivester et al., 2018). In that study, β-glucan concentration was positively correlated with BALF mast cell proportion (Ivester et al., 2018), with each 100 pg/m^3 increase in β-glucan exposure resulting in a 1.3 fold change in mast cell proportions. Despite a difference in β-glucan exposure between groups, the current study did not demonstrate an effect of forage upon BAL mast cell proportions, likely due to the small sample size and the modest difference in exposure between groups. In the current study, though statistically significant, the difference in β-glucan exposure was slightly less than 100 pg/m^3. After 6 weeks eating haylage, horses had a mean BAL mast cell proportion of 2.3. If a 1.3 fold difference in mast cell proportions between groups had occurred due to the increased β-glucan exposure of 100 pg/m^3, the predicted mean mast cell proportion for horses fed hay would be 3%, and a sample size of 35 horses per group would be required to provide 80% power to detect an effect of forage over time. Furthermore, the duration of exposure necessary to affect BAL mast cell proportions is unknown, as the previous study examined horses which had been maintained in the same environment for at

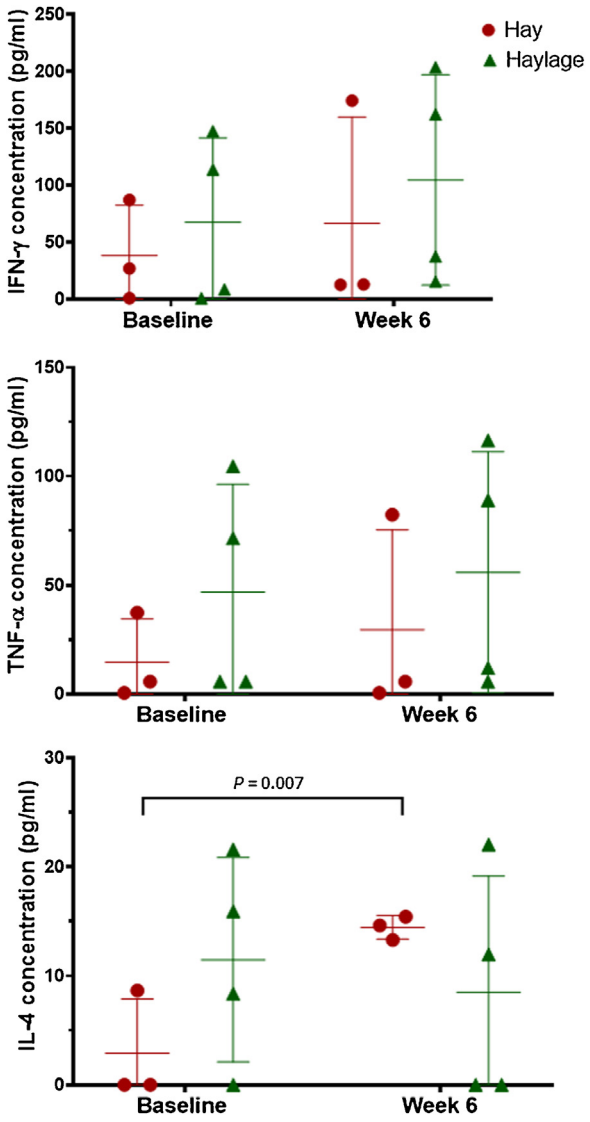

Fig. 5. Mean and standard deviation of bronchoalveolar lavage fluid (BALF) interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-4 (IL-4) concentrations over time in horses fed hay or haylage.

least 60 days. The higher β-glucan exposure of horses eating hay reported in the current study was surprising as it is presumed to reflect higher mold contamination while the hay appeared grossly of good quality.

Endotoxin exposure was not different between horses fed hay or haylage in the present study. The endotoxin exposure measured was lower than in a report of Thoroughbred racehorses fed dry hay while housed on sawdust bedding (7.35 ± 12.8 EU/m^3; Ivester et al., 2018). The concentrations were considerably lower than those previously reported endotoxin exposure of horses eating hay while bedded on straw (7080 EU/m^3) and that of horses on pasture (850 EU/m^3; Berndt et al., 2010). The median endotoxin exposures of both horses eating hay (1.48 EU/m^3) and haylage (1.71 EU/m^3) were lower than those described for young Thoroughbreds in training eating dry hay and bedded with sawdust (59.2 EU/m^3; Ivester et al., 2014b). Endotoxin measurements can be highly variable based upon methods and sample handling (Douwes et al., 2003). The lower endotoxin exposures measured in the current study may relate to the air-conditioned barn or may reflect differences in the hay quality between studies.

Horses eating haylage had a reduction in BALF neutrophil proportions after 2 weeks in the study, and this reduction was still present at week 6, while horses eating hay experienced an increase in BALF neutrophil proportions after 2 weeks. Considering that the horses were kept in the same air space and same bedding, the only differences between the groups was the respirable dust and the β-glucan exposures associated with forage assignment. Findings are consistent with a study showing a positive association between respirable dust and BALF neutrophil proportions such that each 0.01 mg/m^3 increase in respirable dust is predicted to increase BALF neutrophil proportion by 3% (Ivester et al., 2018). Horses presented relatively low BALF proportions at baseline (1.9 ± 1.3%), presumably due to being housed mainly at pasture prior to enrollment. The BALF neutrophil proportions of horses eating hay at week 6 (4.0 ± 0.66%) was consistent with results from Thoroughbred racehorses eating hay while on sawdust bedding (4.8 ± 4.0%; Ivester et al., 2018). Though the mean neutrophil proportion at all time points was considered within the reference range, it is important to consider that even a small increase in the neutrophil proportion can have a negative effect on racing performance (Ivester et al., 2018).

Of the cytokines measured in BALF, only IL-4 concentration differed over time, increasing by week 6 in the horses fed hay, but not in those fed haylage. IL-4 production is a hallmark of a Th2-type response, suggesting the role of aeroallergens in the development of mild equine asthma, as also suggested by the presence of mast cells or eosinophils in the airways of some horses with mild asthma (Lavoie et al., 2011; Beekman et al., 2012). Mast cell inflammation was observed in some of the horses from both groups during the study, but the mean mast cell proportion did not change over time. IL-4 is also a potent activator of neutrophils at the site of inflammation and this activation produces the release of other pro-inflammatory cytokines such as IL-8 that are involved in the influx of more neutrophils to the site of inflammation (Lavoie-Lamoureux et al., 2010). The finding that TNF-α and IFN-γ were not different between groups after 6 weeks was consistent with a previous report (Lavoie et al., 2011), although these cytokines have been related to Th-1 polarization and neutrophilic airway inflammation in racehorses in another study (Richard et al., 2014). It is important to note that for the current study no correction for BALF dilution was made, although this potential confounding factor was likely minimum considering the small variation in BALF volume return compared to the volume instilled. The main limitation of this study was its small sample size. The small number of horses used in the current study may have limited our ability to detect any effect of forage on TNF-α and IFN-γ concentrations. However, the data suggest that there was no effect of forage on BALF cytokine concentrations over time rather than a lack of power to detect an effect. In fact, a sample size calculation showed that in order to detect a difference between baseline and week 6 in TNF-α concentrations, we would need a sample size of 102 horses per group, suggesting minimal effect of forage upon this cytokine.

Overall, the study showed a reduction in airway neutrophilia within 2 weeks of feeding horses with low-dust forage such as haylage. However, this pilot study included only a small number of horses that were kept in a controlled environment therefore, results should be taken with caution and warrant confirmation in subsequent studies involving larger number of horses in field conditions.

Conclusions

Feeding haylage to healthy racehorses in training reduced exposure to respirable dust and β-glucan, resulting in attenuation of airway neutrophilia when compared to horses eating hay. These preliminary findings will require further studies to determine the

effect of low dust forages on lung inflammation in racehorses in natural conditions at the track.

Conflict of interest statement

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

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Effects of forages, dust exposure and proresolving lipids on airway inflammation in horses

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OBJECTIVE
To investigate the role of omega-3 polyunsaturated fatty acids (Ω-3)–derived proresolving lipid mediators (PRLM) in the resolution of mild airway inflammation in horses.

ANIMALS
20 horses with mild airway inflammation.

PROCEDURES
Horses previously eating hay were fed hay pellets (low Ω-3 content; n = 10) or haylage (high Ω-3 content; 9) for 6 weeks. Dust exposure was measured in the breathing zone with a real-time particulate monitor. Bronchoalveolar lavage (BAL) was performed at baseline, week 3, and week 6. The effect of PRLM on neutrophil apoptosis and efferocytosis was examined in vitro. BAL fluid inflammatory cell proportions, apoptosis of circulating neutrophils, efferocytosis displayed by alveolar macrophages, and plasma lipid concentrations were compared between groups fed low and high amounts of Ω-3 by use of repeated measures of generalized linear models.

RESULTS
Dust exposure was significantly higher with hay feeding, compared to haylage and pellets, and equivalent between haylage and pellets. BAL fluid neutrophil proportions decreased significantly in horses fed haylage (baseline, 11.8 ± 2.4%; week 6, 2.5 ± 1.1%) but not pellets (baseline, 12.1 ± 2.3%; week 6, 8.5% ± 1.7%). At week 6, horses eating haylage had significantly lower BAL neutrophil proportions than those eating pellets, and a significantly lower concentration of stearic acid than at baseline. PRLM treatments did not affect neutrophil apoptosis or efferocytosis.

CLINICAL RELEVANCE
Despite similar reduction in dust exposure, horses fed haylage displayed greater resolution of airway inflammation than those fed pellets. This improvement was not associated with increased plasma Ω-3 concentrations. Feeding haylage improves airway inflammation beyond that due to reduced dust exposure, though the mechanism remains unclear.

Mild equine asthma is frequently encountered in performance and pleasure horses,[1–3] and the disease has been related to respirable organic dust exposure.[1,4] Current treatments rely principally on a combination of pharmacological and environmental management. Drugs used to control airway inflammation are mainly glucocorticoids; however, treatment may not be effective,[5] and their use in competing horses is complicated by potential adverse effects and drug residues. Therefore, control and prevention of equine asthma without pharmacologic intervention are key.[6]

The main source of dust exposure to horses is hay, the most commonly used forage in horses.[4] Feeding horses low-dust forages, such as hay pellets or haylage, can decrease dust exposure in the horse's breathing zone by 60% to 70% when compared with hay and by 90% when compared with poor-quality hay, respectively.[7,8] But the resolution of neutrophilic inflammation may require months of low-dust conditions.[9,10] Airway inflammation in human asthmatics is thought to reflect a failure to resolve inflammation, thereby preventing a return to homeostasis after an inflammatory trigger.[11]

The fatty acid composition of an individual's diet influences the formation of inflammatory mediators related to many chronic diseases, such as

asthma in humans.[12,13] Omega-3 polyunsaturated fatty acid (Ω-3) and omega-6 polyunsaturated fatty acid (Ω-6) intake determines cell membrane composition and differences in the dietary intake of these fatty acids can modify downstream production of pro- and anti-inflammatory mediators.[12,14] Proresolving lipid mediators (PRLMs) derived from Ω-3 are central to the resolution of inflammation,[12] at least in part owing to increased apoptosis and clearance of inflammatory neutrophils by efferocytosis.[11] In humans, lipoxin A_4 (LxA4) and resolvin E1 (RvE1) increase neutrophil apoptosis in vitro.[15,16] In murine models of airway inflammation, resolvin D1 (RvD1) decreases neutrophilic inflammation and enhances efferocytosis.[17] To our knowledge, the role of PRLMs in the resolution of airway inflammation in asthmatic horses has not been reported.

Haylage is more abundant in Ω-3, compared with hay[18]; therefore, it may provide additional benefit to equine airway health beyond that of reducing dust exposure. The purpose of this study was to compare airway inflammation and plasma PRLM concentration in horses transitioned from a high-dust, low Ω-3 diet (hay) to a low-dust, high Ω-3 diet (haylage) or low-dust, low Ω-3 diet (hay pellets) and to determine the effect of PRLMs on apoptosis and efferocytosis of equine neutrophils in vitro.

We hypothesized that horses transitioning from a high-dust, low Ω-3 diet to a low-dust, high Ω-3 diet would exhibit a faster resolution of bronchoalveolar lavage fluid (BALF) neutrophilia than horses transitioned to a low-dust, low Ω-3 diet (hay pellets), and that horses fed with the diet high in Ω-3 will have higher plasma concentrations of PRLM. Furthermore, we hypothesized that PRLMs (LxA4, RvD1, and RvE1) would increase the apoptosis of equine neutrophils and their efferocytosis by alveolar macrophages in vitro.

Materials and Methods

Study design

A prospective trial was conducted with 20 healthy horses from the university teaching herd. Fifteen mares and 5 geldings with a mean ± SD age of 16 ± 6 years and a mean weight of 485 ± 41 kg were kept in dry lots and had free access to round bales of hay from covered feeders for at least 6 weeks prior to the start of the study. The horses were housed in 2 separate dry lots at the same facility but did not share a fence line. The study was performed during the winter (February and March 2019), so the dry lots remained free of grass. Horses were not randomized to groups but rather allocated by housing requirements to be fed grass-alfalfa mix haylage (n = 10) or hay pellets (10) for 6 weeks. The horses fed haylage were introduced to the new forage gradually over 7 days. Horses in the hay pellet group were fed timothy-alfalfa mix pellets (DuMOR; Purina Animal Nutrition). Horses were fed forage based on an estimated intake of approximately 2% of body weight/d. They had free access to clean water. Baseline assessment conducted during the period of round bale hay feeding consisted of physical examination, bronchoalveolar lavage (BAL), and blood collection on each horse. Horses were deemed healthy based on a normal physical examination findings and history of normal respiratory health in at least the past 2 years. Sample collection was repeated after 3 and 6 weeks on the new forage (haylage or hay pellets). The Purdue University Animal Care and Use Committee approved all procedures (protocol No. 1111000181).

Clinical score

Horses were trailered to the laboratory located 2 miles from the farm and allowed at least 30 minutes to acclimate before the examination. On physical examination, a clinical score (range, 0 to 21) based on cough, nasal discharge, respiratory efforts, and auscultation was determined as previously described.[19]

Blood collection and processing

Blood was collected by jugular venipuncture with a vacutainer into evacuated tubes containing EDTA. Within 1 hour of blood collection, samples were centrifuged at 1,500 X *g* at room temperature for 10 minutes. Plasma was aspirated carefully and placed into plastic tubes with no additives. Plasma was stored at −80 °C until lipid quantification was performed.

BAL

Horses were sedated by administration of butorphanol (0.02 mg/kg, IV) and xylazine hydrochloride (0.2 to 0.5 mg/kg, IV). A sterile BAL tube (Bivona Medical Technologies; 300-cm length; 10-mm outer diameter) was passed through the nose and wedged into a distal bronchus. Two hundred fifty milliliters of sterile saline (0.9% NaCl) solution was instilled and recovered into 1 aliquot by use of 60-mL syringes. The BALF was immediately placed on ice and processed within 1 hour of collection. Total cell count was measured with a hemocytometer. Cytospin slides were prepared and processed with modified Wright stain. Differential cell counts were determined by enumerating 600 cells/horse by a single individual unaware of forage assignment (CJO). Horses were returned to the farm once recovered from sedation.

Particulate exposure measurements

Exposures to particulate matter (PM) smaller or equal to 1 μm (PM1), PM smaller than 2.5 μm (PM2.5), and PM smaller than 10 μm (PM10) in the horse's breathing zone were measured with a real-time particulate monitor (OPC-N2; Alphasense) for 20 minutes on 2 occasions: when the horses ate dry hay from round bales and while the horses ate the assigned low-dust forage (hay pellets or haylage). The monitor was secured to the crown piece of a breakaway halter, and the inlet of the sampling tube was secured to the noseband of the halter to sample dust at the breathing zone of the horse. The horse was free to move around, eat, and drink as usual **(Supplementary Appendix S1)**.

Plasma lipids quantification

Lipid mediators were analyzed using targeted liquid chromatography–tandem mass spectrometry (LC-TMS) from plasma samples at the Metabolite Profiling Facility, Discovery Park, Purdue University. Five hundred microliters of plasma was transferred to a 5-mL vial and spiked with standards including 500 pg of LxA4-d5 (Cayman Chemical), 500 pg of RvD1-d5 (Cayman Chemical), 2,500 pg of prostaglandin E_2 (PGE_2; PGE_2-d4; Cayman Chemical), and 250 pg of RvE1-d4 (Cayman Chemical). Methanol (2 mL) was added to the spiked samples to extract lipids from the plasma. Samples were vortexed for 1 minute and centrifuged at 20,913 X *g* for 10 minutes to precipitate the proteins. The supernatant was collected and transferred to a new vial to be evaporated and stored at –80 °C until analysis. The dried lipid extracts were reconstituted with 50 µL of methanol and water at a 1:1 volume ratio and submitted for targeted quantification by LC-TMS.[20,21] Ten microliters of the reconstituted sample was delivered to a column (ACQUITY UPLC BEH C18; Waters Corp; 1.7 µm; 2.1 X 100 mm) through a multisampler (G7167B; Agilent Technologies) into a triple quadrupole mass spectrometer (QQQ6470A MassHunter B.06.00; Agilent Technologies) equipped with electrospray ionization jet stream ion source (Agilent Technologies). The binary pump flow rate was set at 0.3 mL/min by use of water and 0.1% formic acid as mobile phase A and acetonitrile and 0.1% formic acid as mobile phase B. The liquid chromatography column was pre-equilibrated for 1 minute with 20% B, and a linear gradient to 100% B was set in 28 minutes and then returned to 20% B in 2 minutes and re-equilibrated for 3 minutes. Concentrations in ng/mL of plasma were obtained by calculating the ratio of the areas of the endogenous and the deuterated internal standard, then multiplied by the concentration of the internal standard. For molecules without deuterated internal standards, calibration curves were done with 5 serial dilutions of the stock solution starting at 100 µg/mL as the highest concentration with a dynamic range and linear ion intensity response (R^2 = 0.99) of the calibration curves were observed for over 4 orders of magnitude. Limit of detection (LOD) and limit of quantification (LOQ) of the analyzed compounds were as follow: palmitic, palmitoleic, stearic, oleic, linoleic acids had an LOD of 1 ng/mL and LOQ of 5 ng/mL; linolenic acid had an LOD of 0.1 ng/mL and LOQ of 1 ng/mL; arachidonic acid, eicosapentaenoic acid (EPA) and DHA had an LOD of 0.01 ng/mL and LOQ of 0.1 ng/mL; PGE_2 and RvD1 had an LOD of 0.1 and 0.05 ng/mL and LOQ of 1 and 0.3 ng/mL, respectively; an LOD of 0.5 ng/mL was obtain for RvE1 and 0.25 ng/mL for protectin D1, maresin 1, LxA4, no LOQ was calculated for these last compounds. Data processing was performed by use of dedicated software (Agilent Technologies).

In vitro assessment of neutrophilic apoptosis

Neutrophils were collected from blood samples obtained by jugular venipuncture and isolated with a discontinuous density gradient (Percoll; Sigma-Aldrich Corp) and underwent centrifugation within 2 hours of collection.[22] One aliquot was used in the efferocytosis assay (described later). The other aliquot of neutrophils was incubated for 30 minutes in culture media only (RPMI-1640; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Hyclone; Thermo Fisher Scientific) as a negative control, or culture media spiked with a PRLM (ie, LXA4 at 100nM, RvE1 at 10nM, or RvD1 at 10nM), or phorbol myristate acetate (Thermo Fisher Scientific) at 100nM as a positive control to cause neutrophil apoptosis at 37 °C in 24-well cell culture plates. Concentrations of PRLMs were selected based on similar studies in other species.[15,16,23] After incubation, the neutrophils were washed and resuspended in PBS solution. Apoptosis was quantified by flow cytometry (CytoFlex; Beckman Coulter) with a fluorescein isothiocyanate annexin V apoptosis detection kit (BD Biosciences) and propidium iodide was used to exclude dead cells following the manufacturer's instructions. Gating was done manually by use of unstained controls and a dedicated software (PlateAnalyzer; Purdue University Cytometry Laboratories). Cells positive for both green and violet stains were considered macrophages that had phagocytosed neutrophils.

In vitro assessment of efferocytosis

Alveolar macrophages were isolated from BALF by cell culture in complete medium (RPMI-1640; Thermo Fisher Scientific) for 4 hours at 37 °C in a humid chamber with 5% CO_2 as previously described.[24] Neutrophils isolated the day before (as already described) were incubated in complete medium (RPMI-1640; Thermo Fisher Scientific) for 16 hours at 37 °C in a humid chamber with 5% CO_2 in 24-well cell culture plates to allow the neutrophils to age and become naturally apoptotic as previously described.[25] After the incubation period, neutrophils were stained green (Celltracker; Thermo Fisher Scientific) and alveolar macrophages stained violet (Tag-it Violet; Biolegend) following manufacturer's instructions. Stained neutrophils were coincubated with stained alveolar macrophages from the same animal in 24-well cell culture plates, at an approximate ratio of 3:1. Cells were counted by use of a hemocytometer to achieve the appropriate ratio. Cells were coincubated and treated with PRLM (LXA4 [100nM], RvE1 [10nM], or RvD1 [10nM]) for 30 minutes at 37 °C with 5% CO_2. Following incubation, cells were washed with PBS solution by use of a 1,000-µL pipette twice to eliminate all the neutrophils that were not phagocytized. Cells were detached from the plate with a cell scraper (Thermo Fisher Scientific). Samples were analyzed by use flow cytometry (CytoFlex; Beckman Coulter), and data analyses were performed with dedicated software (Purdue University Cytometry Laboratories).

Statistical analysis

An a priori sample size calculation indicated that a sample size of 20 horses (10 horses/forage group) would provide 80% power to detect a clinically relevant difference of 5% in BALF neutro-

phil proportions between groups at a significance level of $\alpha = 0.05$. Data analyses and graphs were performed with various computer software packages (ProcGLIMMIX version 9.4; SAS Institute; GraphPad Prism version 8.4.2; GraphPad Software Inc; MetaboAnalyst version 3.0; Wishart Research Group). An exploratory analysis of correlations between the various lipid mediators, age, BALF neutrophil proportion, and BALF total nucleated cell count at baseline was performed by calculating Spearman rank correlations with unadjusted *P* values. Generalized linear models were constructed to examine the effect of forage assignment on BALF cytology over time and to compare in vitro neutrophil apoptosis and efferocytosis between forage groups and in vitro treatments. All models were controlled for age. Tukey-adjusted values of $P < 0.05$ were considered significant.

Results

Horses

One horse from the haylage group was removed from the study because of the development of pneumonia during study day 42 (week 6). Both feeding protocols were well tolerated by the horses, and no adverse effects were observed during the study. The mean age (95% CI) of the horses from the pellet and haylage groups was 17.4 (14.4 to 20.4) years and 14.9 (11.9 to 17.9) years, respectively. Clinical score (mean [95% CI]) was not different between groups at baseline (pellet = 3.9 [2.8 to 5.5]; haylage = 5.2 [3.8 to 7.1]; $P = 0.541$) or week 6 (pellet = 1.9 [1.0 to 3.5]; haylage = 4.6 [3.1 to 7.0]; $P = 0.083$; **Supplementary Appendix S2**) but was significantly ($P = 0.025$) lower in the horses fed pellets at week 6 than at baseline.

Dust exposure

Breathing-zone measurements of PM1, PM2.5, and PM10 were available for 8 horses when eating from hay in round bales. Four of these horses were subsequently assigned to the hay pellet group, and 4 were assigned to the haylage group. Breathing-zone measurements of exposure were obtained for 10 horses while eating pellets and 9 horses eating haylage. Dust exposures to PM1, PM2.5, and PM10 in the horses' breathing zone were significantly higher when horses were eating round bales of hay (baseline measurement; $P < 0.005$) than when consuming hay pellets or haylage (week 6 measurement; **Figure 1**) but were not different between horses eating pellets and haylage ($P > 0.055$; **Supplementary Appendix S3**).

Airway cytology

Horses eating hay from round bales exhibited a mild degree of neutrophilic airway inflammation (BALF neutrophils = 12%).[6] Horses fed haylage experienced a marked and gradual decrease in BALF neutrophil proportions between baseline and week 6 ($P = 0.002$), while horses fed pellets experienced a mild but nonsignificant decrease ($P = 0.283$; **Figure 2**; **Supplementary Appendix S4**). At week 6, horses eating haylage had significantly lower BALF neutrophil proportions than those eating pellets ($P = 0.014$). BALF cytology data for macrophages, lymphocytes, mast cells, and eosinophils showed no effect of time or forage **(Table 1)**.

Plasma lipid quantification

The only quantifiable PRLM was RvD1. The other targeted PRLMs, including RvE1, LxA4, ma-

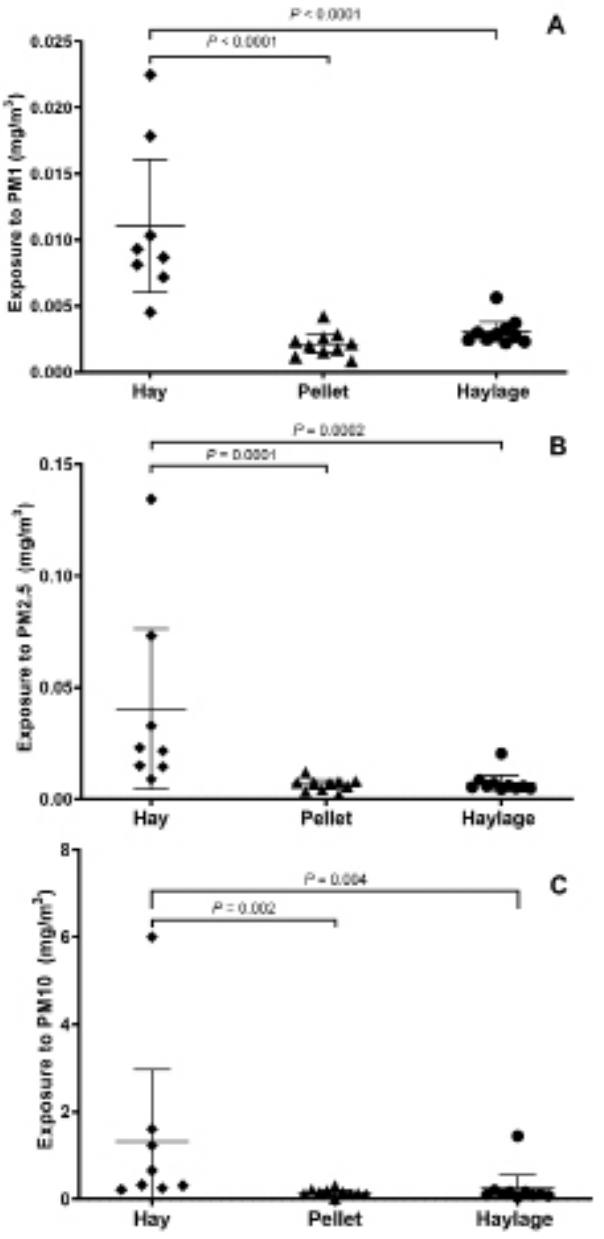

Figure 1—Scatterplot of dust exposure in horses breathing zone while eating hay, pelleted hay, and haylage. A—Particulate matter (PM) concentration smaller or equal to 1 μm (PM1) concentration. B—PM concentration smaller than 2.5 μm (PM2.5). C—PM concentration smaller than 10 μm (PM10). Horizontal bars indicate mean and 95% CI.

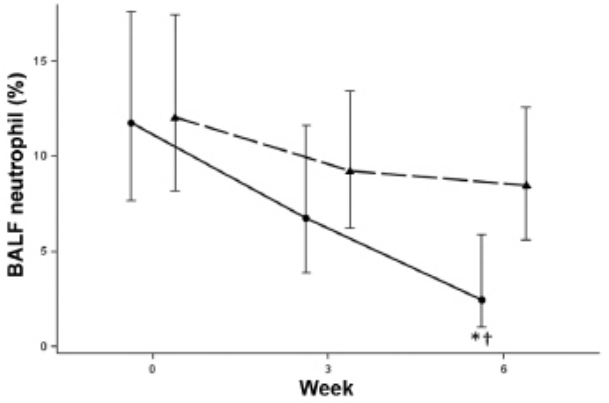

Figure 2—Comparison of the mean neutrophil proportion in bronchoalveolar lavage fluid (BALF) between horses fed pelleted hay (triangles) and haylage (circles) over 6 weeks. Horizontal bars indicate 95% CI. *Significantly different from pellet group at week 6 (P = 0.014). †Significantly different from baseline (P = 0.0017).

Table 1—Bronchoalveolar lavage fluid (BALF) differential cell count from horses fed round bale hay for at least 6 weeks (baseline; n = 19) and after being fed timothy-alfalfa mix pellets (10) or grass-alfalfa mix haylage (9) for 3 and 6 weeks. Data are presented as mean ± SD.

Variable	Time	Pellet	Haylage
BALF mast cell proportion (%)	Baseline	2.0 ± 0.4	1.9 ± 0.4
	Week 3	1.9 ± 0.4	2.1 ± 0.3
	Week 6	2.0 ± 0.3	1.5 ± 0.3
BALF eosinophil proportion (%)	Baseline	0.5 ± 0.2	0.2 ± 0.2
	Week 3	0.1 ± 0.1	0.3 ± 0.2
	Week 6	0.2 ± 0.2	0.1 ± 0.1
BALF macrophage proportion (%)	Baseline	37 ± 3.4	43 ± 3.8
	Week 3	38 ± 2.9	41 ± 3.7
	Week 6	39 ± 3.0	53 ± 3.5
BALF lymphocytes proportion (%)	Baseline	49 ± 2.6	42 ± 2.7
	Week 3	51 ± 2.3	50 ± 2.8
	Week 6	50 ± 2.3	42 ± 2.6

resin 1, and protectin D1, were under the LOD (0.5 ng/mL). No effect of forage or time was observed on RvD1, arachidonic acid, EPA, docosahexaenoic acid (DHA), oleic acid, linoleic acid, palmitoleic acid, palmitic acid, or PGE_2 (P > 0.250; **Supplementary Appendix S5**). Stearic acid concentration in plasma was affected by forage over time (P = 0.046), with a significant decrease at week 6 in horses eating haylage when compared with baseline (P = 0.048; **Figure 3**). Linolenic acid concentration decreased over time regardless of forage assignment (P = 0.010).

There was a negative correlation between PGE_2 and the age of the horses (r_s[16] = –0.486; P = 0.041). Both DHA and EPA were positively correlated to each other, and DHA was negatively correlated with BALF total nucleated cell count **(Supplementary Appendix S6)**. Plasma concentrations of palmitoleic acid, palmitic acid, oleic acid, linoleic acid, arachidonic acid, and stearic acid were significantly correlated **(Supplementary Appendix S7)**.

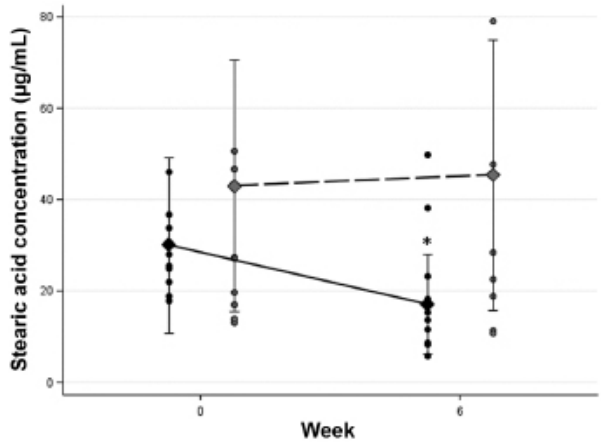

Figure 3—Mean and 95% CI of plasma stearic acid concentration per group over time. Black solid line = Haylage group. Circles = Observations. Diamonds = Mean. Error bars = 95% CI. Gray dashed line = Pellet group. *Significantly different from baseline (P = 0.04).

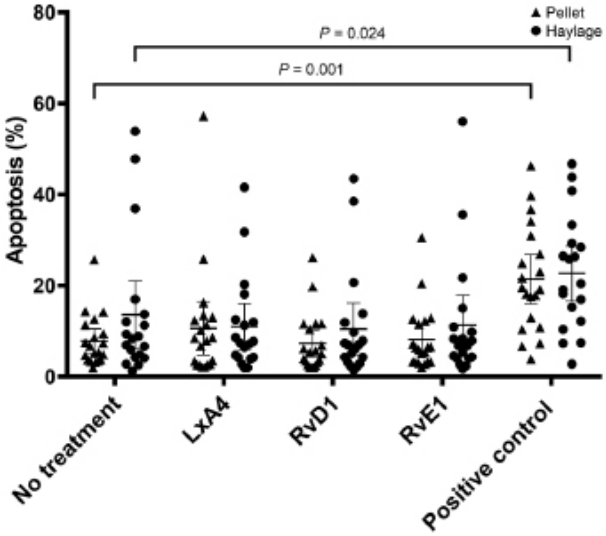

Figure 4—Scatterplot of neutrophil apoptosis (%) with baseline and week 6 data pooled. Horizontal bars indicate mean and 95% CI. Neutrophils were incubated in media only (no treatment) or media spiked with proresolving lipid mediators (lipoxin A4 [LxA4], resolvin D1 [RvD1], or resolvin E1 [RvE1]) or phorbol mysteristate acetate to cause neutrophil apoptosis (positive control).

In vitro determination of apoptosis and efferocytosis

Apoptosis did not differ between forage groups (P = 0.496) or time points (P = 0.624), nor did it vary with any in vitro PRLM treatment (P > 0.950). The only effect of treatment was observed with an increase in apoptosis with phorbol myristate acetate (positive control), compared with effects of no treatment in both groups (P < 0.001; **Figure 4**; **Supplementary Appendix S8**).

Overnight incubation in culture media resulted in apoptosis in roughly 50% of isolated neutrophils (fluorescein isothiocyanate–annexin V apoptosis

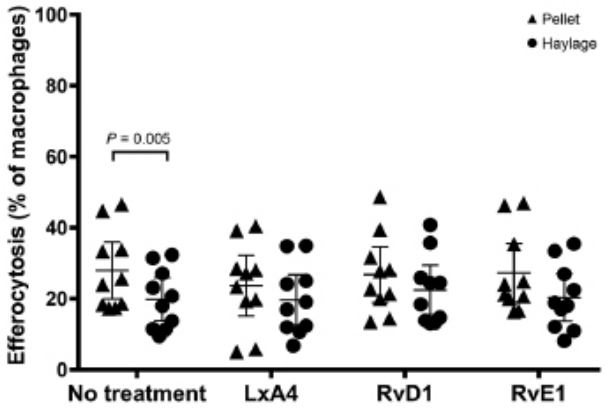

Figure 5—Scatterplot of efferocytosis (%) at week 6. Horizontal bars indicate mean and 95% CI. Neutrophils were coincubated with alveolar macrophages in media only (no treatment) or media spiked with proresolving lipid mediators (lipoxin A4, resolvin D1, or resolvin E1). ***See*** Figure 4 for remainder of key.

positive) for use in the efferocytosis assay. Efferocytosis was significantly higher in the horses eating pellets than in the horses eating haylage ($P = 0.005$; **Figure 5**; **Supplementary Appendix S9**). There was no difference in efferocytosis between PRLM treatments ($P > 0.334$).

Discussion

The purpose of this study was to examine the role of dust exposure and lipid mediators in the resolution of neutrophilic airway inflammation when horses transitioned from high-dust to low-dust forages. Another objective was to gain insight into the mechanism of resolution of airway neutrophilia by studying the effect of PRLMs on apoptosis of equine neutrophils and efferocytosis of neutrophils by alveolar macrophages in vitro. We found that horses fed hay from round bales were exposed to high levels of dust (PM1, PM2.5, and PM10) in the breathing zone, and this exposure was associated with mild neutrophilic airway inflammation. Transitioning the horses' diet from hay to haylage resulted in a marked decrease in airway neutrophilia over 6 weeks. Only a mild decrease in airway neutrophilia, albeit not statistically significant, was observed in horses fed hay pellets for 6 weeks, despite a similar reduction in dust exposure as horses fed haylage. Stearic acid was the only lipid mediator measured that differed between horses fed haylage and pellets. Contrary to our hypothesis, efferocytosis was higher in horses eating hay pellets, compared with horses eating haylage, and we found no evidence of any direct effect of PRLM treatments on apoptosis or efferocytosis in vitro.

As anticipated, breathing-zone measurements of PM1, PM2.5, and PM10 were not different between low-dust forages, and both pelleted hay and haylage resulted in significantly lower dust exposure when compared with results for the round bale hay. Exposure to PM2.5 and PM10 has been related to visible tracheal mucus score in racehorses.[26] Similarly, PM10 has been associated with tracheal mucus score and also neutrophil counts in tracheal wash cytology in racehorses.[27] In humans, PM2.5 has been associated with an increase in the prevalence and morbidity of respiratory diseases such as lung cancer and asthma.[28] As expected, PM10 measured at the breathing zone of horses eating round bales (0.12 mg/m^3) was higher than results reported for stabled Thoroughbreds eating hay (0.073 mg/m^3).[27]

Clinical score did not differ between groups at baseline or week 6, but it was significantly lower at week 6 in horses fed pellets when compared to the same group at baseline. The decrease in clinical score was due to changes in abdominal lift and nostril flare. These scores ranged from 0 (absent) to 1 (mild) in all horses, regardless of forage assignment. With the exception of one, all horses in the pellet group had a score of 0 assigned to both abdominal lift and nasal flare at week 6; however, this finding was unlikely to be clinically relevant and the decrease in clinical score in the group fed pellets was presumably related to lower stress level or part of random variation. Future studies with a larger number of horses will be needed to confirm this result. All horses displayed mild BALF neutrophilia at baseline. These horses were kept on dry lots during the winter and fed round bales of hay for at least 6 weeks prior to the study. Similar results have been described in a population of horses eating round bales of hay during winter, in which 80% of the horses presented with a mild to moderate increase in BALF neutrophil proportions; however, dust exposure was not measured.[29] After 6 weeks on low-dust diets, both groups of horses in the present study showed a decrease in BALF neutrophil proportions, but this decrease was only statistically and clinically significant in horses eating haylage. In previous studies, horses[9,10] with severe equine asthma exacerbation placed on a low-dust diet (pasture and pelleted feed) demonstrated an improvement in BAL neutrophilia, but only after 2 to 6 months. Horses with severe equine asthma kept on grass pasture, a diet naturally higher in Ω-3 than dried forages,[18] may exhibit resolution of BALF neutrophilia within 2 months.[30] Similarly, in another study,[19] horses with severe asthma eating a low-dust diet (complete pelleted diet) and an Ω-3 supplement for 2 months display significantly greater improvement in clinical signs and BAL neutrophilia when compared with a group that received the low-dust diet with a placebo supplement.[19] Taken together, these results suggest that the presumed higher content in Ω-3 of the haylage fed in the present study contributed to the rapid resolution of BALF neutrophilia, similar to that seen with Ω-3 supplementation and access to pasture.

We were unable to detect most of the targeted PRLMs using LC-TMS. This technique has been used to measure plasma concentrations of PRLM in humans and rodents,[31,32] but the detection of these molecules remains challenging.[33] These mediators display bioactivity at concentrations in the picomolar and lower nanomolar ranges.[34] The PRLMs such as LXA4, RvD1, RvE1, maresin 1, and protectin D1 may have been present at concentrations and activ-

ity that differed between forage groups but were below our LOD. We were able to measure only plasma concentrations of RvD1 from the horses in this study, and 20 samples were under the LOD (50 pg/mL). The mean plasma concentration at baseline in the horses was 142 ± 169 pg/mL; this concentration is higher than those previously described in humans (24.4 ± 2.5 pg/mL).[31,35] The RvD1 concentration did not change with the haylage, despite the presumed higher content of Ω-3. The failure to detect a statistical difference between groups is likely due to the high variability of the measurements and relatively small sample size. The only plasma lipid affected by forage was stearic acid, which decreased significantly with the consumption of haylage. Stearic acid is a saturated fatty acid present in forages[36] such as hay and has been reported to increase as forages become more mature.[37] A possibility is that pellets were made from a more mature alfalfa-grass hay mixture as compared with haylage. Stearic acid has been associated with inflammatory processes such as osteoarthritis and obesity in humans. In vitro studies indicate a proapoptotic effect of stearic acid on macrophages[38] and the capacity to enhance the production of oxygen radicals by neutrophils.[39] Stearic acid was correlated with oleic acid and arachidonic acid, which are lipid mediators associated with inflammatory processes in humans.[40] Thus, compared with pellets, the effect of haylage on stearic acid concentration may reflect decreased dietary intake of this lipid, a potential role of this molecule in airway inflammation, or both.

Prostaglandin E_2, a fatty acid that is derived from arachidonic acid, was negatively correlated with age at baseline. The opposite has been reported in humans and mice with an increase in PGE_2 production seen with aging. This increase in synthesis has been related to various chronic diseases in elderly humans, such as arthritis, and cancer.[41] On the other hand, PGE_2 has been described to prevent allergen-induced bronchoconstriction and to reduce airway hyper-responsiveness and inflammation in bronchial asthma in humans.[42] In horses with severe asthma, diminished production of PGE_2 by airway mucosa has been reported,[43] while increased BALF PGE_2 concentration has also been found.[44] In the present study, plasma PGE_2 concentrations decreased after 6 weeks in the horses fed haylage, but the change did not reach statistical significance. Again, the lack of statistical significance could be related to the small sample size and the high variability of the measurements.

Decreased neutrophilic airway inflammation over the 6-week course of the study as a result of low-dust exposure was expected to be secondary to enhanced neutrophil apoptosis and efferocytosis mediated by PRLMs. Horses fed haylage did indeed exhibit improved resolution of BAL neutrophilia, but we did not find increased neutrophil apoptosis nor increased efferocytosis in this group. Instead, horses on the low Ω-3 pellet diet demonstrated greater alveolar macrophage efferocytosis, potentially indicating that alveolar macrophages maintained an activated state in horses fed pellets due to continued inflammation, while resolution of inflammation in the haylage group resulted in a more quiescent population of alveolar macrophages.[45] Neither apoptosis of neutrophils harvested from the systemic circulation nor efferocytosis of apoptotic neutrophils by alveolar macrophages was affected by PRLM treatments at the concentrations and incubation times used in this study. PRLMs have been reported to affect both apoptosis and efferocytosis in vitro in other animal models and humans.[13,15–17,46] The concentrations of the PRLM and incubation times used in the present study were reportedly effective in previous studies with rodent or human cells,[15,16] but may not have been sufficient for equine cells. Another potential explanation is that multiple PRLMs are needed to work in concert. Alternatively, other PRLMs such as maresins[47] may be more important in resolving inflammation in the horse. It is also possible that the accelerated resolution of inflammation observed in the horses fed haylage in this study was mainly due to inhibition of transendothelial migration of neutrophils to the lung by PRLMs.[11] Such an effect would not be apparent with the type of in vitro studies performed in this study.

A limitation of this study was that the groups were made by convenience and not randomized. Other unobserved differences between the groups may have obscured the effect of forage in this study. This latter possibility is unlikely since horses were studied at the same time and were kept in paddocks in close proximity. However, a randomized, crossover study design would have been best to reduce confounding factors. Also, as previously noted, the plasma lipid detection method may not have provided the necessary sensitivity to detect differences in PRLM between the groups, as evidenced by our inability to detect most of the targeted molecules. Larger sample size may have enabled us to detect an effect of forage on those molecules we were able to measure. Finally, Ω-3 and Ω-6 are important components of cell walls.[48] In other species, such as felines[49] and humans,[50] DHA and EPA have been detected in RBCs at higher concentrations than in plasma. Therefore, measuring the concentration of these molecules in RBCs, rather than plasma, could be considered for future studies.

In conclusion, horses transitioning from feeding round bale hay to haylage and pelleted hay had a comparable reduction in dust exposure, but only those fed haylage for 6 weeks experienced a significant reduction in BALF neutrophilia. This clinical effect was not accompanied by an in vitro effect of PRLM treatments on neutrophil apoptosis or efferocytosis. The reduction in neutrophilic airway inflammation exhibited by horses fed haylage is greater than expected by the decreased dust exposure alone; however, the mechanism remains unclear.

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ORIGINAL ARTICLE

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Validation of a flow cytometric assay to detect intraerythrocytic reactive oxygen species in horses

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Abstract

Background: Oxidative stress refers to the accumulation of reactive oxygen species (ROS). Most assays for ROS detection are costly, laborious, and usually use indirect markers. The use of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) is a possible alternative. This substance becomes a fluorochrome when oxidized by ROS, with the resultant fluorescence proportional to ROS concentration. Erythrocytes are highly exposed to ROS, resulting in cell damage and consequently impaired oxygen delivery. The effects of this exposure in physiologic and pathologic conditions necessitate an improvement in ROS detection methods.

Objective: We aimed to validate intraerythrocytic ROS detection by flow cytometry using DCHF-DA in healthy horses.

Methods: Erythrocytes from 31 healthy horses were isolated, incubated with DCFH-DA, and either left unstimulated or stimulated with hydrogen peroxide (H_2O_2). For specificity, each cellular component of blood was separated and plotted according to its size and complexity. Samples were run in triplicate for intra-assay precision and five consecutive times for inter-assay repeatability. Stability was determined by analyzing the same sample up to 48 hours after blood collection. The acceptable coefficient of variation (CV) was ≤20%.

Results: The intra-assay CV was 1.7% and 13.3%, and the inter-assay CV was 4.8% and 17.8% for unstimulated and stimulated samples, respectively. Unstimulated and stimulated samples were stable for up to 48 and 24 hours, respectively. Stimulated samples had greater fluorescence than unstimulated samples ($P < .0001$).

Conclusions: This flow cytometric assay demonstrated adequate specificity, precision, and stability and is, therefore, a promising technique with multiple applications for studying oxidative stress in horses.

KEYWORDS

DCFH-DA, equine, flow cytometry, free radical

1 | INTRODUCTION

Reactive oxygen species (ROS) are a group of unstable, reactive molecules derived from molecular oxygen. They are generated continuously in all aerobic cells as a by-product of metabolism or deliberately produced during phagocytosis and cell signaling. Reactive oxygen species can induce cellular senescence and apoptosis.[1,2] The family of ROS includes free radicals such as superoxide anion ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), and hydroxyl radical ($^{\bullet}OH$), as well as reactive compounds, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and lipid radicals.[3] When in excess, ROS cause the oxidation of biological molecules, such as nucleic acids, proteins, and lipids, with consequent cell damage.[2]

Cellular defense mechanisms have evolved to mitigate free radical formation. These mechanisms use enzymes that inactivate or scavenge free radicals. The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx). When ROS generation exceeds the antioxidant capacity of the cell, oxidative stress occurs.[4] Oxidative stress has been implicated in the pathogenesis of several human conditions, such as cardiovascular disease, atherosclerosis, reperfusion injury, neurologic disease, the response to drugs, and cancer.[4] In horses, increaased systemic and intraerythrocytic oxidative stress have been reported after intense exercise[5] and with theileriosis.[6] However, horses with Pituitary Pars Intermedia Dysfunction (PPID)[7] and recurrent airway obstruction do not present with systemic oxidative stress.[8] The total antioxidant capacity is reduced in horses with rhabdomyolysis[9] and pyometra.[10]

Erythrocytes are particularly susceptible to oxidative injury because of continuous exposure to endogenous and exogenous sources of ROS. Auto-oxidation of hemoglobin to methemoglobin and $O_2^{\bullet-}$ represent endogenous sources of ROS production, while exogenous sources include neutrophils, macrophages, and endothelial cells.[11] The absence of nuclei in mature erythrocytes makes them even more susceptible to oxidative injury, given their inability to synthesize new proteins in response to oxidative insults.[12] Compared with human erythrocytes, hemoglobin in equine erythrocytes is more susceptible to oxidation, possibly due to a "weaker" antioxidant system and, therefore, methemoglobin formation, making them more sensitive to oxidative damage and hemolysis.[13] Erythrocyte oxidative stress has been implicated in erythrocytic lifespan shortening and impairing cellular deformability needed for effective oxygen transport and delivery to tissues.[11]

Given the instability and short half-life of ROS, measurement of these molecules in biological samples is challenging.[14] Sensitive techniques to detect ROS directly are lacking. Investigators usually depend on indirect markers of oxidative damage (eg, measuring antioxidant substances and enzyme activity).[4] Flow cytometry has been successfully used in human medicine for directly labeling intracellular ROS in a variety of cells using the fluorescent marker 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA).[4,15] DCFH-DA is cell-permeable and readily crosses erythrocyte membranes. Inside the cell, DCFH-DA is cleaved by intracellular esterases, forming a membrane-impermeable, polar molecule called 2'7'-dichlorodihydrofluorescein (H_2DCF). Subsequent H_2DCF oxidation by intracellular ROS produces a highly fluorescent product called 2',7'-dichlorofluorescein (DCF), detectable at an excitation wavelength of 488 nm by flow cytometry.[15,16] The objective of this study was to validate the use of DCFH-DA for the detection of ROS in equine erythrocytes from healthy horses.

2 | MATERIAL AND METHODS

2.1 | Animals and samples

We included 31 adult horses (eight Thoroughbreds, eight Quarter Horses, five Standardbreds, two Appaloosas, two Tennessee Walking Horses, two mixed breed horses, and one each of Arabians, Pintos, Paint Horses, and Saddlebreds), determined to be healthy by standard physical examinations and unremarkable laboratory data (complete blood count and biochemistry panel, Data S1). The average age was 16 ± 7.5 years. The animals were obtained from the Purdue University Veterinary Teaching herd after approval by the Purdue Animal Care and Use Committee (1 712 001 658).

We collected whole blood samples from the jugular vein of each horse, immediately transferred into EDTA vacuum tubes (BD Vacutainer, Thermo Fisher Scientific, Waltham, MA, USA), and stored at 4°C until analysis (within two hours of collection). All samples were allocated into four groups: unstimulated vehicle control, consisting of dimethyl sulfoxide (DMSO, American Bioanalytical, Natick, MA, USA) and phosphate saline buffer [PBS pH 7.4; 137mM NaCl (EMD Chemicals Inc, Savannah, GA, USA), 10mM Na_2HPO_4 (Sigma-Aldrich, St Louis, MO, USA), 2.7mM KCl (Mallinckrodt Specialty Chemicals Co., Saint Louis, MO, USA), and 1.8mM KH_2PO_4 (Mallinckrodt Specialty Chemicals Co.)]; stimulated vehicle control, consisting of DMSO and H_2O_2 (Sigma-Aldrich); unstimulated DCFH-DA (Sigma-Aldrich), consisting of DCFH-DA and PBS; and stimulated DCFH-DA, consisting of DCFH-DA and H_2O_2. Stimulation with H_2O_2 aimed to promote the formation of ROS within the erythrocytes. Unstimulated samples received PBS at the same volume of H_2O_2. The vehicle used to dilute DCFH-DA was DMSO.

Ten microliters of DMSO (vehicle control) or a solution of 500 μM DCFH-DA was added to 5 mL round-bottom tubes (Corning Falcon Round-Bottom Polypropylene Tubes, Corning Incorporated, Corning, NY, USA). The blood samples were centrifuged (Sorvall Legend X1R, Thermo Fisher Scientific, Waltham, MA, USA) at 3,000*g* for 5 minutes at 4°C for removal of plasma and buffy coats. Ten microliters of erythrocytes was diluted in 5 mL of PBS supplemented with 1% w/v bovine serum albumin (PBSA, Fisher Scientific, Pittsburg, PA, USA). One hundred microliters of erythrocyte solution was added to the respective tubes, followed by incubation at 37°C for 20 minutes. After incubation, 10 μL of PBS was added to the unstimulated cells, and 10 μL of a solution of 20 mM H_2O_2 was added to the stimulated cells. After a 20-minute incubation at room temperature (RT), the samples were quenched with 300 μL PBSA

1% and immediately analyzed by flow cytometry (Accuri C6 flow cytometer, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Reactive oxygen species-dependent fluorescence was detected by green fluorescence with an excitation wavelength of 488 nm (FL1 channel) with gating around erythrocytes only.

2.2 | Validation

The validation methods and strategies used in our study followed guidelines and recommendations specific for flow cytometric assays.[17] We determined specificity at three levels, precision, and stability, as well as baseline values for healthy horses.

2.3 | Specificity assays

2.3.1 | Specificity level one

Because DCFH-DA is not cell-specific, the fluorescence specificity of erythrocytes was determined based on correct gating and appropriate removal of leukocytes and platelets. For this purpose, we collected three EDTA whole blood samples from three different horses.[17] We obtained platelet-rich plasma (PRP) after the blood was allowed to settle for 20 minutes at RT, followed by centrifugation of the plasma at 300*g* for 10 minutes at RT. We removed the buffy coat from a second EDTA sample and then transferred the buffy coat to an erythrocyte lysis solution at 10x the volume (pH 7.4; 0.15mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, all from Sigma-Aldrich) to obtain the leukocytes. After incubation in a rocker for 10 minutes at RT, the sample was centrifuged at 3,000*g* for 5 minutes at RT, and we washed the pellet with the same volume of lysis solution previously used. The incubation and centrifugation were repeated, and the pellet resuspended in 2 mL PBSA 1%. We obtained an erythrocyte solution with the third EDTA sample, as described in the previous section. Samples from every horse were analyzed in duplicate and allocated as described above (unstimulated versus stimulated, vehicle vs DCFH-DA) for flow cytometric gating. The gates were saved as a template and used to analyze all remaining samples in the study (Figure 1A).

2.3.2 | Specificity level two

To evaluate whether cellular fluorescence reflects ROS generation, a series of triplicate samples from three different horses[17] were incubated with a solution of 5 mM sodium azide (NaN_3, Sigma-Aldrich) for 10 minutes after incubation with a vehicle or DCFH-DA to ensure that the cellular fluorescence reflected ROS generation. The erythrocytes were then stimulated with H_2O_2 (as described above) or remained unstimulated.

2.3.3 | Specificity level three

Level three determined the assay specificity in a dose-dependent manner.[17] Samples from three different horses were incubated with 0 (vehicle only), 1, 5, 10, 50, or 100 μM DCFH-DA. Secondly, samples were incubated with increasing concentrations of H_2O_2 from 0 (PBS) to 6 mM with 2 mM increments. The percentage of positive cells and the median fluorescence intensity (MFI) were analyzed.

2.4 | Precision and stability assays

Precision was determined based on the intra-assay (within-run) and inter-assay (between subjects) repeatability. We ran stimulated and unstimulated samples and samples with and without DCFH-DA (as described above) from three different horses in triplicate for intra-assay precision and five different analytical runs for inter-assay precision. We considered as acceptable a coefficient of variation (CV) ≤20% for both assays.[17]

The capability of the samples to retain the initial measurements over time was determined by measuring MFI at 3, 6, 24, 36, and

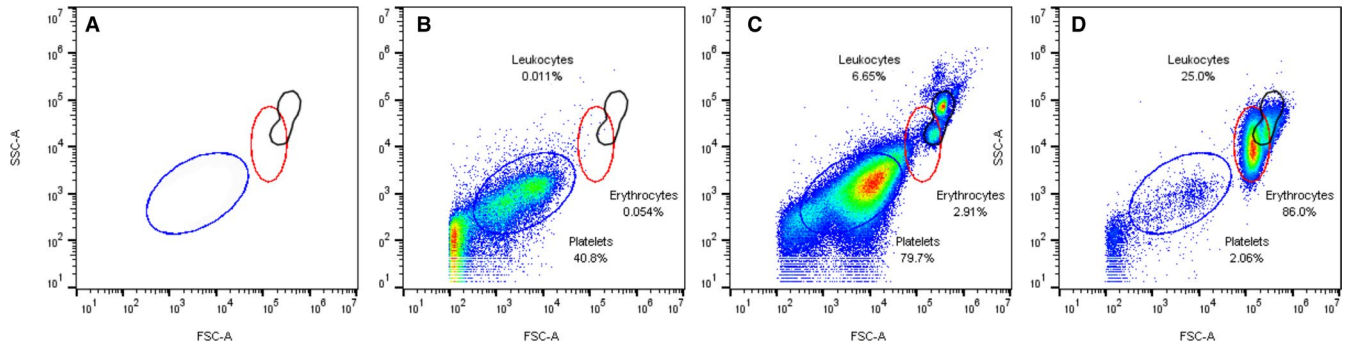

FIGURE 1 Logarithmic forward (FSC-A) and side scatter plots (SSC-A) showing the gating strategy using one representative sample. (A) Depiction of all gates, where grey represents leukocytes, red, erythrocytes, and blue, platelets. (B) Platelet-rich plasma sample, containing mainly platelets (within blue gate represented in figure A). (C) Buffy coat sample containing leukocytes (within grey gate represented in figure A) and most likely platelets and remaining erythrocytes. (D) Erythrocyte sample, containing mainly erythrocytes and small numbers of remaining platelets and leukocytes

48 hours postcollection of three different horses. The samples were kept refrigerated (4°C) at all times. We considered a CV of ≤20% to be acceptable for each time postcollection compared with baseline.[17]

2.5 | Baseline

The assay was performed in 31 horses to establish a response pattern and baseline quantity of intraerythrocytic ROS.

2.6 | Statistical analysis

We collected at least 50,000 events in the erythrocyte gate for each validation phase. Data were exported from BD Accuri C6 Software as FCS files and analyzed with FlowJo 10.5.3 (FlowJo, Ashland, OR, USA) and Excel 2007 (Microsoft Office, Redmond, WA, USA) software. Using GraphPad Prism 8.0.2 (GraphPad, San Diego, CA, USA), the statistical analysis consisted of the Shapiro-Wilk normality test followed by the Wilcoxon signed-rank test assessing the percentage of positive cells and the paired MFI t-test for specificity level two. Additionally, we applied a repeated measures two-way ANOVA ($P < .05$) with Geisser-Greenhouse correction, followed by Tukey's test to compare stimulated and unstimulated samples for specificity level three. Nonlinear regression using a robust fit method of normalized data was used to compare the increasing DCFH-DA concentration in the group with 6mM H_2O_2 only. The formula below was used for CV calculations of both precision and stability, where ln(10) is the natural log of 10, and σ is the standard deviation.

$$CV(\%) = 100\% \sqrt{10^{\ln(10)\sigma^2} - 1}$$

3 | RESULTS

3.1 | Specificity

3.1.1 | Specificity level one

In the PRP samples, the platelets were small events in logarithmic scale (Figure 1B), and they were observed forming a narrowed cloud of events in the corresponding logarithmic forward (FSC-A) and side scatter plots (SSC-A). In the leukocyte samples, the leukocytes were the largest events. It is possible to observe an overlap within the erythrocyte cloud and a large number of platelets and small cellular fragments generated during the lysis procedure (Figure 1C). Finally, the isolated erythrocyte samples provided one single cloud of events above the platelets (Figure 1D) that had a mild overlap with leukocytes (presumably lymphocytes, the smallest leukocytes present in the blood). We assessed the cellularity of the preparations of each cell type after cytocentrifugation (600 rpm, 5 min, Shandon CytoSpin 3, Thermo Fisher Scientific, Waltham, MA, USA). They showed rare leukocyte contamination in the PRP and erythrocyte solution (mainly lymphocytes), and moderate contamination of platelets (approximately 3-5 platelets per leukocyte, evaluated in 10 oil-immersion 100x fields) and partially lysed erythrocytes (1-3 erythrocytes per leukocyte, evaluated in 10 oil-immersion 100x fields) in the leukocyte preparation (Figure S1).

3.1.2 | Specificity level two

The percentage of cells positive for green fluorescence in both unstimulated and stimulated samples was highly variable and not statistically different despite preincubation with NaN_3 (Table 1). However, the MFI was significantly increased in both unstimulated and stimulated samples after incubation with NaN_3 ($P = .0044$ and 0.0456, respectively).

3.1.3 | Specificity level three

The DCFH-DA concentration effect is statistically significant for both positivity ($P = .0009$, Table 2) and MFI ($P < .0001$, Table 2), but not for the H_2O_2 concentration. Thus, the lower H_2O_2 concentration was used in further experiments. For regression analysis, we observed that the fluorescent signals were not linear with the DCFH-DA concentrations (Figure 2). On multiple comparison analyses of the different DCFH-DA concentrations using 2 mM H_2O_2, there were no differences ($P > .05$) between 50 and 100 μM DCFH-DA. Also, there was no difference between the 5, 10, and 50 μM concentrations. Based on these findings, we chose to use 50 μM in further experiments, given the higher fluorescence signal.

TABLE 1 The median percentage of cells (range) positive for 2′-7′-dichlorofluorescein, and the median fluorescence intensity (MFI) (mean ± standard deviation) in erythrocytes preincubated with or without 5 mM sodium azide (NaN_3) and either stimulated with 2 mM H_2O_2 or unstimulated

	Percentage positive cells	Median fluorescence intensity		
	Unstimulated	Stimulated	Unstimulated[a]	Stimulated[a]
Without NaN_3	0.2 (0.2 - 0.3)	58.6 (53.1 - 66.2)	1.523 ± 0.023	2.896 ± 0.127
With NaN_3	0.1 (0.1 - 0.4)	81.40 (59.6 - 81.5)	1.585 ± 0.026	3.035 ± 0.130

[a]Indicate statistical difference between samples incubated with or without NaN_3 ($P < .05$).

TABLE 2 Percentage of positive cells and median fluorescence intensity (MFI) (mean ± standard deviation) of erythrocytes incubated with increasing concentrations of H_2O_2 (columns, 0, 2, 4, and 6 mM) and increasing concentrations of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA, rows, 0, 1, 5, 10, 50, and 100 μM)

	0mM H_2O_2		2mM H_2O_2		4mM H_2O_2		6mM H_2O_2	
	%	MFI	%	MFI	%	MFI	%	MFI
0μM DCFH-DA	0.1 ± 0.001[a]	1.926 ± 0.008[a]	N/A	N/A	N/A	N/A	0.9 ± 0.066[a]	2.264 ± 0.017[a]
1μM DCFH-DA	N/A	N/A	9.3 ± 1.888	2.385 ± 0.011	7.3 ± 2.177	2.353 ± 0.042	5.7 ± 1.544	2.336 ± 0.015
5μM DCFH-DA	N/A	N/A	29.4 ± 3.832	2.527 ± 0.016	27.4 ± 4.888	2.509 ± 0.024	24.1 ± 2.686	2.482 ± 0.007
10μM DCFH-DA	N/A	N/A	37.4 ± 4.336	2.584 ± 0.018	38.1 ± 6.358	2.589 ± 0.039	38.2 ± 4.922	2.587 ± 0.030
50μM DCFH-DA	N/A	N/A	71.3 ± 4.692	2.947 ± 0.074	73.8 ± 2.217	2.986 ± 0.025	75.0 ± 2.751	3.004 ± 0.029
100μM DCFH-DA	0.4 ± 0.212[a]	1.991 ± 0.012[a]	88.0 ± 3.792	3.296 ± 0.133	86.5 ± 4.573	3.253 ± 0.084	91.6 ± 2.157	3.415 ± 0.074

Note: N/A: Nonapplicable indicates combinations of H_2O_2 and DCFH-DA concentrations that were not performed.
[a]Parameters that were not evaluated in multiple comparisons.

3.2 | Precision and stability

The intra-assay CV was 1.7% in unstimulated cells, while this value increased to 13.3% in stimulated cells (Table 3). The same pattern was observed in the inter-assay precision. The inter-assay coefficient of variation was 4.8% and 17.8% for unstimulated and stimulated cells, respectively, with high inter-individual variance (Table 4).

In the stability assay, although the samples retained the baseline measurement for up to 48 hours after blood collection when kept at 4°C (the maximum CV was 5.6% in comparison to baseline), the stimulated samples were only stable for up to 24 hours after blood collection (Table 5).

3.3 | Baseline

The assay was performed on all 31 horses to establish a response pattern. The scatter data and median logarithmic MFI are shown in Figure 3.

4 | DISCUSSION

In our study, we successfully validated the measurement of intraerythrocytic ROS in healthy horses with DCFH-DA using flow cytometry. Assessing intracellular ROS levels in erythrocytes can potentially contribute to understanding the effect of free radical accumulation during exercise[18,19] and disease.[20] Since DCFH-DA is not cell-specific, the same technique can be easily adapted to the erythrocytes of other species or any other single-cell suspension by adjusting the gating. Although we observed a mild overlap in the gating strategy adopted between erythrocytes and leukocytes, the centrifugation step substantially reduced the number of leukocytes in the sample, as assessed by the cytocentrifuge preparations. We believe that the numbers of leukocytes were too low to interfere with the data, mainly because the majority of leukocytes in the gate are lymphocytes, which have the lowest peroxidase activity among leukocytes.[21]

To establish parameters to verify the correct fluorescence intensity, samples containing only the vehicle (DMSO) used to dilute DCFH-DA were analyzed to set auto-fluorescence. The samples stimulated with H_2O_2 were used as a positive control for fluorescence and to measure erythrocytic antioxidant capacity indirectly. The fluorescence intensity evaluated in unstimulated cells reflects the current ROS levels. Overall, we observed a one-unit increase on a logarithmic scale between unstimulated and stimulated cells. In contrast with our findings, this difference was not observed in human erythrocytes in a previous study.[15] We attribute this discrepancy to be related to the use of a linear scale to evaluate fluorescence, which is likely less sensitive to detect minor changes, and to the instrumentation (BD FACSCalibur). The equipment used in our study (BD Accuri C6) has digital signal processing with higher laser efficiency.[22] Also, the cited study used methanol, a cell fixative, to

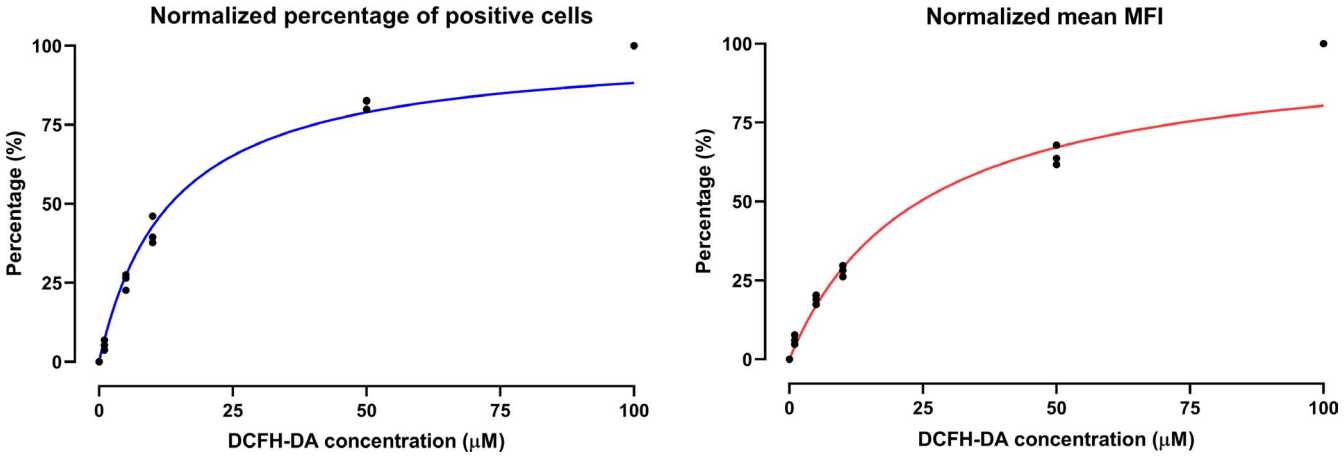

FIGURE 2 Nonlinear robust fit curves of normalized percentage cells positive for 2′-7′-dichlorofluorescein (left blue graph) and average median fluorescence Intensity (MFI, right red graph) with an incremental concentration of 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 0, 1, 5, 10, 50, 100μM) stimulated with 6 m M H_2O_2 evaluated by flow cytometry. The curves are similar but generated different best-fit values

TABLE 3 The mean intra-assay coefficient of variation (CV) of the median fluorescence intensity (MFI) from three horses in each of the five runs and in both conditions (unstimulated and stimulated cells) and the mean CV per condition

	Unstimulated					Stimulated				
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 1	Run 2	Run 3	Run 4	Run 5
CV (%) per run	2.1	0.7	2.6	1.3	1.9	8.4	17.3	14.3	6.7	17.7
CV (%) per condition	1.7					13.3				

TABLE 4 The mean and standard deviation of the inter-assay median fluorescence intensity (MFI) for each horse, mean inter-assay coefficient of variation (CV) of each horse, and mean inter-assay CV per condition (unstimulated and stimulated cells)

	Unstimulated			Stimulated		
	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
Mean MFI	1.954	1.971	1.976	2.926	2.840	2.911
Standard deviation	0.016	0.067	0.027	0.067	0.107	0.055
CV (%)	3.7	4.6	6.1	15.6	25.1	12.8
Mean CV (%)	4.8			17.8		

dilute the fluorochromes, which could have inhibited, at least partially, the influx of DCFH-DA into the cells.

Sodium azide was used in the specificity assay to demonstrate that the production of fluorescence is directly linked with ROS production. Sodium azide inhibits catalase, an enzyme responsible for the transformation of H_2O_2 into H_2O and O_2.[23] It was expected that the presence of NaN_3 would cause a marked increase in the MFI and in the percentage of cells positive for DCF since H_2O_2 would accumulate in cytoplasm and react with H_2DCF. One study shows that catalase is more effective than glutathione peroxidase for scavenging exogenous H_2O_2, making the NaN_3 a reliable strategy to link the specificity of DFCH-DA to the fluorescence observed.[24] In the same study, the auto-fluorescence of degraded hemoglobin was detected by flow cytometry, and the addition of 1mM NaN_3 increased the fluorescence approximately 14-fold after 72 hours of incubation.[24] In our study, the increase was one-fold after 10 minutes of incubation with 5mM NaN_3 and the addition of exogenous H_2O_2. Similar effects were observed previously in human erythrocytes.[15] We found that the percentage of DCF positive cells was less reliable than the MFI to evaluate the degree of oxidation given the more pronounced variability in the percentage of positive cells, as observed in the calculated standard deviations (Tables 2 and 3).

In the third level of specificity, higher H_2O_2 concentrations were not increasing the MFI but damaging the cells and causing visible shattering in the flow acquisition plots (Figure S2). For this reason, we believed that evaluating the percentage of DCF-positive cells would not provide as much robust and reliable information as could

TABLE 5 The means of the logarithmic median fluorescence intensity (MFI), standard deviation (SD) of unstimulated and stimulated cells at baseline, and 3, 6, 24, 36, and 48 h after blood collection, and the coefficients of variation (CVs) at each time in comparison to baseline

	Unstimulated			Stimulated		
	Mean MFI	SD	CV (%)	Mean MFI	SD	CV (%)
Baseline	1.937	0.006	N/A	2.855	0.047	N/A
3 h	1.945	0.003	1.3	2.921	0.066	10.8
6 h	1.940	0.011	0.6	2.949	0.051	15.4
24 h	1.952	0.014	2.5	2.927	0.123	11.7
36 h	1.957	0.025	3.3	3.030	0.112	29.0
48 h	1.971	0.006	5.6	3.014	0.047	26.2

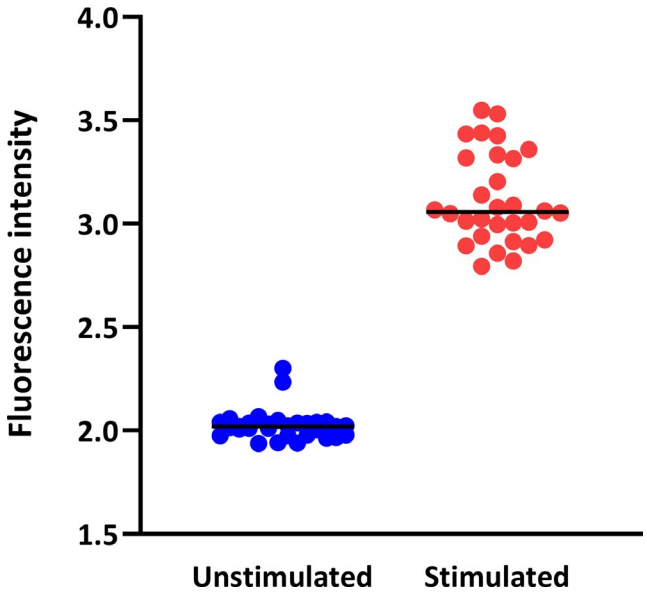

FIGURE 3 Individual logarithmic median fluorescence intensities (MFI) in unstimulated and stimulated samples from 31 horses showing a significant statistical difference (Wilcoxon signed-rank test, $P < .0001$) between groups are plotted in the graph. The horizontal bars represent the medians

be achieved with MFI since the latter was proportional to intracellular ROS concentrations. The DCFH-DA concentration curve indicated that the amount of the chemical was not linear and reached a plateau, most likely representing intracellular saturation. Therefore, selecting the best concentration for both fluorochrome and the stimulator is recommended for each different cell and species studied.

In both inter-assay and intra-assay precision evaluations, the variation among the stimulated samples was consistently increased compared with the unstimulated samples. Other than individual factors (eg, age, sex, nutrition, and genetics), this could be explained by the variability between horses in the proportions of young and aged erythrocytes that can contain different amounts of remaining antioxidant enzymes during a cell's lifespan. Recent studies evaluating the omics (ie, metabolomics, proteomics, and lipidomics) of stored human erythrocytes have demonstrated that ROS are involved in many aspects of cell damage during storage.[25] These insights might help elucidate cellular interactions during pathologic states.

Given the labile nature of ROS, formation and neutralization occur dynamically. Thus, determining ROS in biological samples would not be reliable. However, catalase activity, for instance, remains relatively unchanged after 72 hours when kept at 4°C, but decreases 38% if kept at 25°C.[24] Therefore, the reduced temperature in which the samples were stored immediately after blood collection most likely reduced the entire cellular metabolism to minimal levels. In human leukoreduced stored erythrocytes, ROS concentration reaches its highest point within the first two weeks of storage.[25] In our study, the amount of ROS in unstimulated samples remained stable for up to 48 hours, while the amount of ROS produced in stimulated samples tended to increase over time, most likely because the intracellular enzymes were neutralizing the free radicals present and there was no de novo enzymatic synthesis in erythrocytes. These data allow investigators to plan shipping samples or batch analyses, adding flexibility to experimental designs if kept refrigerated and stored for no longer than 24 hours.

As potential limitations of our study, accuracy was not measured; since there is no reference standard for ROS measurements, making flow cytometric comparisons with other methods not possible. Also, we did not establish a lower ROS detection limit in cells with this method. We also did not evaluate intra-laboratory variability since the same investigator analyzed all samples.

In conclusion, we have validated a flow cytometric-based assay for the detection of intraerythrocytic ROS in horses. The assay is specific for erythrocytes based on gating and the correct separation of cells. It is also precise with maximum intra- and inter-assay CVs of 13% and 18%, respectively. Stability was excellent for the unstimulated samples; however, the stimulated samples were only stable for up to 24 hours after collection, after which intracellular antioxidants were presumably depleted. The use of DCFH-DA to detect equine intraerythrocytic ROS with flow cytometry is a promising technique with multiple applications to study oxidative stress in horses.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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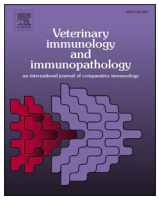

The effect of lower airway inflammation on inflammatory cytokine gene expression in bronchoalveolar lavage fluid and whole blood in racing Thoroughbreds

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ABSTRACT

Background: Immunological mechanisms involved in the pathogenesis of mild to moderate equine asthma (MEA) are not completely understood. There are limited data on bronchoalveolar lavage fluid (BALF) and blood inflammatory cytokine profiles in racehorses with MEA, and the effect of racing on inflammatory cytokines is unknown.

Hypothesis/objectives: We hypothesized that inflammatory cytokine gene expression in BALF and resting blood would be higher in racehorses with lower airway inflammation compared to healthy controls, and that gene expression in blood collected immediately post-race would be increased compared to resting blood in racehorses with lower airway inflammation.

Animals: 38 racing Thoroughbreds (samples: 30 resting blood, 22 post-race BALF, 41 post-race blood).

Methods: Prospective observational study. Inflammatory cytokine gene expression was determined in resting blood, post-race BALF and post-race blood from racehorses with lower airway inflammation and controls.

Results: Lower airway inflammation was diagnosed in 79 % of racehorses (23 % neutrophilic, 67 % mastocytic, and 10 % mixed). There was no difference in gene expression in BALF or resting blood between racehorses with lower airway inflammation and controls. IL-8 gene expression was higher in post-race blood compared to resting peripheral blood, regardless of disease ($p = 0052$). BALF neutrophil proportions increased with increasing IL-1β gene expression in all sample types ($p = 0.0025$). BALF mast cell proportions increased with increasing TNF-α gene expression in post-race blood ($p = 0.015$).

Conclusions and clinical importance: Lower airway inflammation was common in a population of racehorses without respiratory signs or exercise intolerance. Exercise alone increased peripheral blood *IL-8* gene expression. Inflammatory cytokine gene expression was not increased in BALF or resting blood in horses with subclinical lower airway inflammation, precluding its diagnostic utility in clinical practice.

1. Introduction

Mild to moderate equine asthma (MEA), previously known as inflammatory airway disease, is a relatively common syndrome in poorly performing horses that is characterized by non-infectious lower airway inflammation (Ivester et al., 2018; Orard et al., 2016). Varying BALF cytological profiles among affected horses indicate differing disease mechanisms, although airway hypersensitivity to inhaled irritants is common (Couetil et al., 2016; Ivester et al., 2014; Lavoie et al., 2011; Nolen-Walston et al., 2013).

Possible lower airway inflammatory phenotypes based on BALF cytology include BALF neutrophilia, mastocytosis, eosinophilia, and

Abbreviations: BALF, bronchoalveolar lavage fluid; B-GUS, beta glucuronidase; MEA, mild to moderate equine asthma; SEA, severe equine asthma; WBC, white blood cell.

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mixed inflammation (Bedenice et al., 2008; Lavoie et al., 2011). Among these different phenotypes, the mechanisms of disease and the immune response in affected horses is not completely understood. Both innate and adaptive immunity play a role in the pathogenesis of allergic airway disease. In an attempt to elucidate the immune response in horses with MEA, BALF gene expression of several inflammatory cytokines has been investigated (Beekman et al., 2012; Hughes et al., 2011; Lavoie et al., 2011; Riihimaki et al., 2008; Ryhner et al., 2008). Cytokines of interest include those produced by innate immune cells, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6 and IL-8, as well as effector T cells including interferon-gamma (IFN-γ), IL-2 (T helper [Th] 1), IL-4, IL-5 (Th2) and IL-17 (Th17). Among horses with MEA, increases in BALF TNF-α and IL-1β gene expression are common, but inconsistent results have been reported for expression of IL-6, IL-8 and IL-17 (Beekman et al., 2012; Hughes et al., 2011; Lavoie et al., 2011). Increases in TNF-α and IL-1β gene expression suggest induction of an innate immune response, which is consistent with the influx of innate immune cells into BALF in affected horses (Laan et al., 2006; Richard et al., 2014). The inconsistent patterns in BALF cytokines that are involved in neutrophil chemotaxis and activation, including IL-8 and IL-17, likely reflect the various airway inflammatory phenotypes (Beekman et al., 2012; Hughes et al., 2011). In fact, there is little data comparing inflammatory cytokine gene expression among horses with different phenotypes (Beekman et al., 2012; Lavoie et al., 2011).

Intense exercise in healthy horses has been shown to downregulate the innate pulmonary immune response and upregulate the innate systemic immune response (Frellstedt et al., 2014). However, the effect of racing on inflammatory cytokine gene expression in BALF from horses with MEA is unclear, given that previous studies measured BALF cytokine gene expression or concentration at rest (i.e. $\geq$ 12 h post-exercise) (Beekman et al., 2012; Hughes et al., 2011; Lavoie et al., 2011; Richard et al., 2014). In humans, moderate to high-intensity exercise has been shown to upregulate gene expression and serum concentrations of innate inflammatory cytokines within 1 h of exercise completion (La Gerche et al., 2015; Ribeiro-Samora et al., 2017; Van Pelt et al., 2017; Zaldivar et al., 2006). It is possible that inflammation associated with MEA might be exacerbated immediately following intense exercise, and be reflected by BALF and peripheral blood inflammatory cytokine expression profiles. In fact, inflammatory cytokine gene expression in peripheral blood from horses with MEA, either at rest or post-exercise, has not been reported.

Therefore, the objectives of this study were to compare inflammatory cytokine gene expression in BALF and peripheral blood in racehorses with lower airway inflammation and healthy controls, determine the effect of intense exercise (racing) on gene expression in peripheral blood from racehorses with lower airway inflammation, and further define cytokine expression profiles among different lower airway inflammation phenotypes. We aimed to test the following two hypotheses: 1) Inflammatory cytokine gene expression in BALF and resting peripheral blood will be higher in racehorses with lower airway inflammation compared to healthy racehorses; and 2) Inflammatory cytokine gene expression in peripheral blood collected within 1 h of race completion will be increased compared to resting peripheral blood in horses with lower airway inflammation.

2. Materials and methods

2.1. Study design

A prospective observational study was performed as part of a larger study investigating the impact of airway inflammation upon race outcomes in Thoroughbred horses racing in Indiana during three race meets between September 2014 and October 2016 (Ivester et al., 2018). Horses were recruited through communication with trainers and owners, and informed consent was obtained prior to racing. Horses were eligible for re-enrollment with each race entered. This study was approved by the Institution's Animal Care and Use Committee and the Indiana Horse Racing Commission.

2.2. Sample collection

Each horse enrolled in the study underwent jugular venipuncture, endoscopic examination and BAL within 1 h of race completion. Ten mL whole blood was collected and immediately placed in specialized RNA stabilization tubes (Tempus™ Blood RNA Tubes, ThermoFisher Scientific Inc., Waltham, MA) for subsequent gene expression analysis; this blood sample was designated "post-race." Horses were restrained with a nasal twitch and a flexible fiber-optic endoscope (7.9 mm outer diameter, 100 cm in length) was passed through the ventral meatus to the level of the pharynx and then advanced into the trachea until the tracheal bifurcation was visible. Scores were assigned from 0 to 4 for tracheal mucus accumulation (Gerber et al., 2004) and exercise induced pulmonary hemorrhage (EIPH) (Hinchcliff et al., 2005). To facilitate BAL, the carina and larynx were sprayed with a 0.4 % lidocaine solution (20 – 30 mL at each site) as the endoscope was removed. For BALF collection, horses were sedated with xylazine hydrochloride (0.2 – 0.5 mg/kg IV) and butorphanol tartrate (0.02 mg/kg IV), and 250 mL of sterile 0.9 % NaCl were infused through a sterile BAL tube (300 cm long, 10 mm outer diameter; Bivona Medical Technologies, Gary, IN), followed by manual recovery with 60 mL sterile syringes as previously described (Ivester et al., 2018). Approximately 100 mL BALF were centrifuged at 300 *g* for 10 min. Cells were resuspended in 3 mL 0.9 % NaCl and transferred into specialized RNA stabilization tubes (Tempus™ Blood RNA Tubes, ThermoFisher Scientific Inc., Waltham, MA) for subsequent gene expression analysis. A second whole blood sample was collected 4–7 days after race completion to determine "resting" gene expression analysis. Resting BALF samples were not obtained due to the uncertainty of race schedules and trainer reluctance given the possible deleterious effects of recent BALF testing on racing performance since the procedure induces an influx of neutrophils in airways lasting at least 48 h (Sweeney et al., 1994). All samples were stored at −20 °C prior to processing.

2.3. Case definition

Lower airway inflammation was diagnosed if BALF differential cytology counts were > 5% neutrophils, > 2% mast cells, > 1% eosinophils, or any combination thereof (Couetil et al., 2016). Horses were classified with neutrophilic, mastocytic, eosinophilic or mixed (> 1 WBC type increased) inflammation.

2.4. Gene expression analysis

RNA was isolated using a benchtop purification instrument (KingFisher Flex System, ThermoFisher Scientific Inc., Waltham, MA) and nucleic acid purification kit (MagMAX™ CORE Nucleic Acid Purification Kit, Applied Biosystems, Beverly, MA) per the manufacturer's recommendations, except there was no DNase step and the pelleted RNA was re-suspended in 600 μl viral lysis buffer (ThermoFisher Scientific Inc., Waltham, MA). RNA was reverse transcribed using the Maxima H Minus Mastermix (ThermoFisher Scientific Inc., Waltham, MA) and a Veriti thermal cycler (Applied Biosystems, Beverly, MA) per the manufacturer's recommendations. Gene expression was determined using the relative quantitation method (Livak and Schmittgen, 2001) and positive and negative control samples were included. Averaged delta-CT results for each gene from all resting peripheral blood samples were used as the calibrator for each respective gene to allow for comparison of all samples against the entire study population. β-glucuronidase (β-GUS) was used as the reference gene for all samples (Breathnach et al., 2006; Page et al., 2017) and samples were assayed in duplicate using commercially available exon-spanning primers and probes (ThermoFisher Scientific Inc., Waltham, MA):

- β-GUS (Ec03470630_m1)
- TNF-α (Ec03467871_m1)
- IL-1β (Ec04260298_s1)
- IL-6 (Ec03468678_m1)
- IL-8 (Ec03468860_m1)
- IL-17c (Ec03470096_m1)

2.5. Data analysis

Relationships between post-race and resting cytokine expression in peripheral blood were first examined with scatter plots and evaluated by calculating the Spearman rank correlations. Relative cytokine expression was compared between sample type (BAL versus peripheral blood), time of collection (post-race versus resting) and airway inflammation subgroup (healthy, neutrophilic, mastocytic, eosinophilic, and mixed inflammation) with generalized linear mixed models with lognormal distribution, including the random effect of horse to account for repeated measures. Type I error rate was controlled for post-hoc pairwise comparisons using the Tukey-Kramer adjustment for multiple comparisons. The relationship between inflammatory cell proportions and relative cytokine gene expression was examined using generalized linear mixed models using the logit link and including the random effect of horse. All analyses were controlled for degree of EIPH by including endoscopic EIPH score assigned at race completion as a fixed effect in each model. Analyses were performed using statistical software (Proc GLiMMIX in SAS 9.4, Cary, NC). Significance was set at $P < 0.05$.

3. Results

A total of 38 horses were included in the study with 4 horses enrolled twice (42 sample sets). All horses had a normal respiratory examination and none were exercise intolerant. The mean age of enrolled horses was 4.0 ± 1.3 years. Endoscopic EIPH scores ranged from 0 to 4, with a median score of 1 (interquartile range = 2). EIPH score did not significantly affect any cytokine gene expression ($P > 0.15$). Endoscopic mucus scores ranged from 0 to 5 with a median score of 1 (interquartile range = 3). Of the 38 horses included in the study, lower airway inflammation was diagnosed based on BALF cytology in 30 (79 %). Of these, 7 (23 %) had neutrophilic inflammation, 20 (67 %) mastocytic inflammation, and 3 (10 %) mixed inflammation. No horse had eosinophilic inflammation. The mixed inflammation group exhibited a higher proportion of neutrophils (9.6 %) than the neutrophilic inflammation group (6.8 %), although this difference was not statistically significant (p = 0.096). Time constraints present when sampling multiple horses on the same race day prevented processing of every BALF specimen for subsequent gene expression analysis, and horses were not always available for collection of resting blood samples. Therefore, gene expression analysis was available for 22 BALF samples, 41 post-race peripheral blood samples, and 30 resting peripheral blood samples.

Relative expression of inflammatory cytokines in post-race peripheral blood was significantly correlated with that found in resting blood samples except in the case of IL-17 (Fig. 1). There was no difference in inflammatory cytokine gene expression in BALF between horses with lower airway inflammation and healthy horses (TNF-α: p = 0.83; IL-1β: p = 0.65; IL-6: p = 0.97; IL-8: p = 0.85; IL-17: p = 0.63). There was no difference in inflammatory cytokine gene expression in resting peripheral blood between horses with lower airway inflammation and healthy horses (TNF-α: p = 0.57; IL-1β: p = 0.24; IL-6: p = 0.67; IL-8: p = 0.97; IL-17: p = 0.51).

Gene expression of TNF-α, IL-1β, IL-6 and IL-17 in peripheral blood did not differ between post-race and resting samples (p > 0.25); however, IL-8 expression was higher in post-race peripheral blood samples compared to resting peripheral blood samples (p = 0.0052), regardless of whether lower airway inflammation was present or not (Fig. 2).

When asthma phenotype was considered, inflammatory cytokine gene expression in post-race and resting peripheral blood samples did

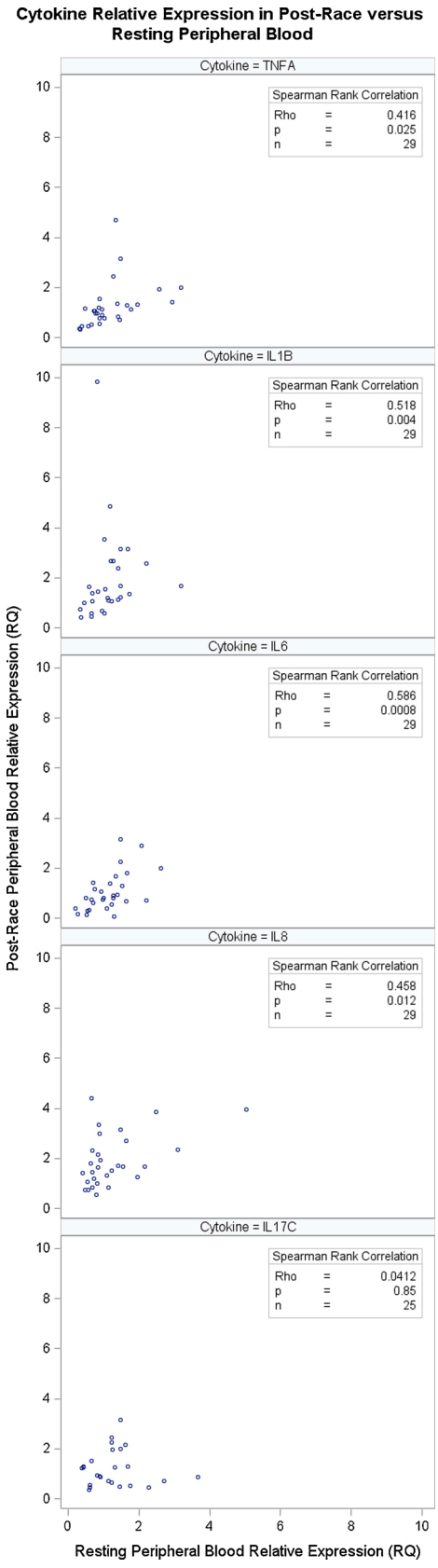

Fig. 1. Scatter plots and Spearman rank correlations between relative gene expression in post-race and resting peripheral blood samples.

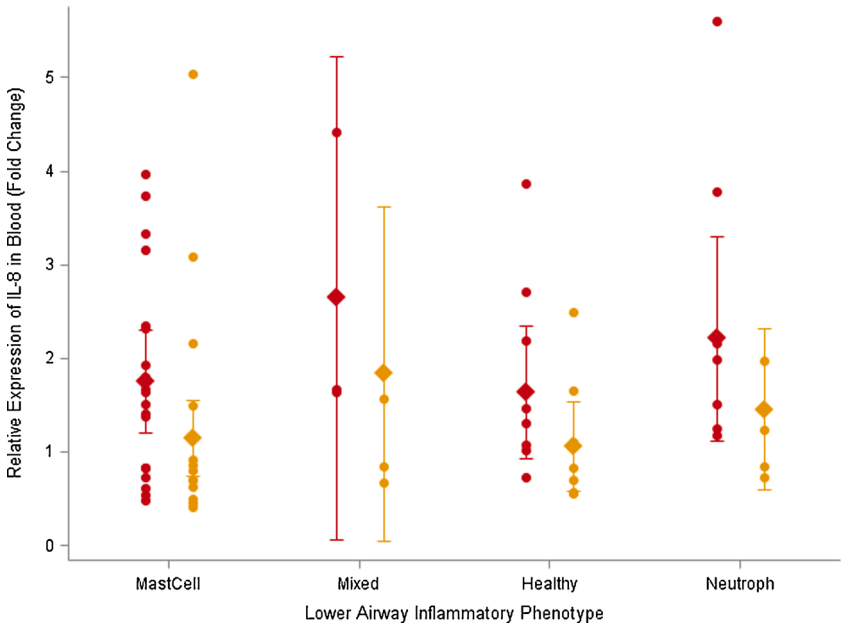

Fig. 2. Relative gene expression of IL-8 in peripheral blood samples by lower airway inflammation phenotype. Red: post-race peripheral blood sample; orange: resting peripheral blood sample; diamond: mean; whiskers: 95 % confidence interval of the mean; circles: observations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

not vary among inflammatory phenotype groups for TNF-α ($p = 0.83$), IL-1β ($p = 0.41$), IL-8 ($p = 0.78$), or IL-17 ($p = 0.48$). However, relative expression of IL-6 was lower in post-race peripheral blood in the mixed inflammation group when compared to healthy horses ($p = 0.02$), horses with mast cell inflammation ($p = 0.0008$) and horses with neutrophilic inflammation ($p = 0.0039$); Fig. 3).

BALF neutrophil proportions increased with increasing IL-1β expression ($p = 0.0025$), regardless of sample type (Fig. 4). When sample type was considered, BALF neutrophil proportions increased with increasing IL-1β expression in BALF ($p = 0.003$), post-race peripheral blood ($p = 0.018$) and resting peripheral blood ($p < 0.0001$).

BALF mast cell proportions increased with increasing TNF-α expression ($p = 0.015$), regardless of sample type. When sample type was considered, BALF mast cell proportions increased with increasing TNF-α expression in post-race peripheral blood ($p = 0.002$; Fig. 5), but not BALF ($p = 0.24$) or resting peripheral blood ($p = 0.12$).

4. Discussion

In this population of racing Thoroughbreds without respiratory clinical signs or exercise intolerance, lower airway inflammation was not associated with increased expression of the BALF or resting peripheral blood inflammatory cytokine genes examined here. Previous studies have reported an upregulation in BALF inflammatory cytokine

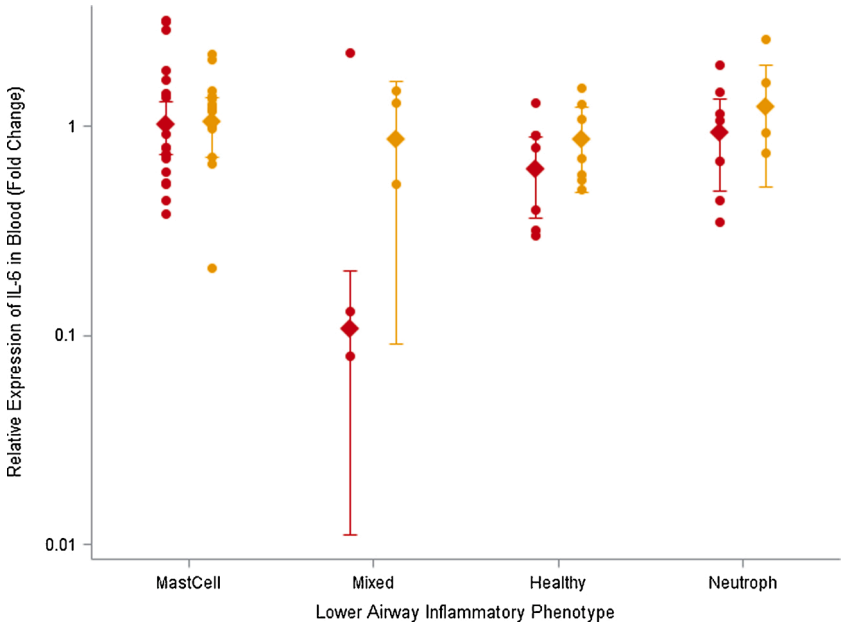

Fig. 3. Relative gene expression of IL-6 in peripheral blood samples by lower airway inflammation phenotype. Red: post-race peripheral blood sample; orange: resting peripheral blood sample; diamond: mean; whiskers: 95 % confidence interval of the mean; circles: observations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

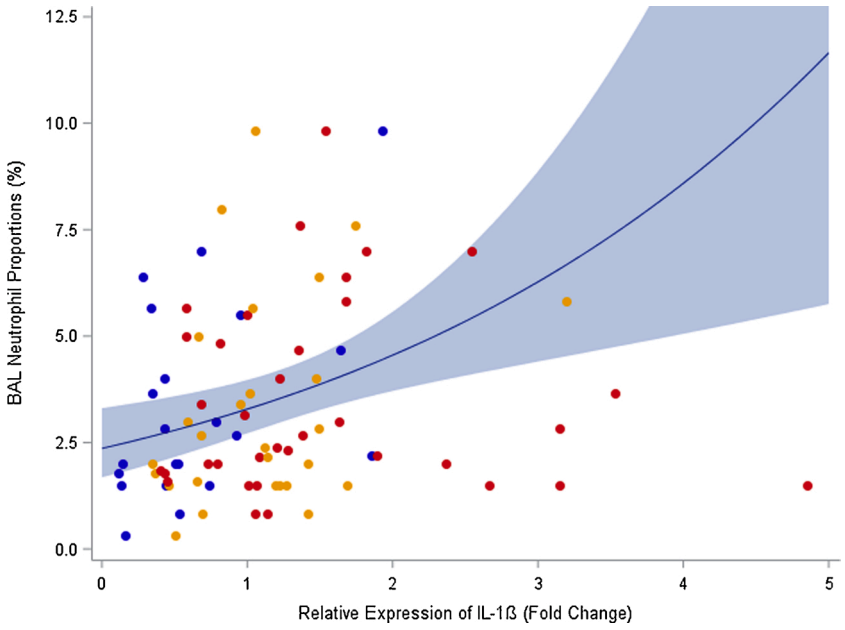

Fig. 4. Generalized linear mixed model of BAL neutrophil proportions versus IL-1β gene expression (all samples). Blue: post-race BAL sample; red: post-race peripheral blood sample; orange: resting peripheral blood sample; solid line: predicted mean response; band: 95 % confidence interval of mean response; circles: observations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

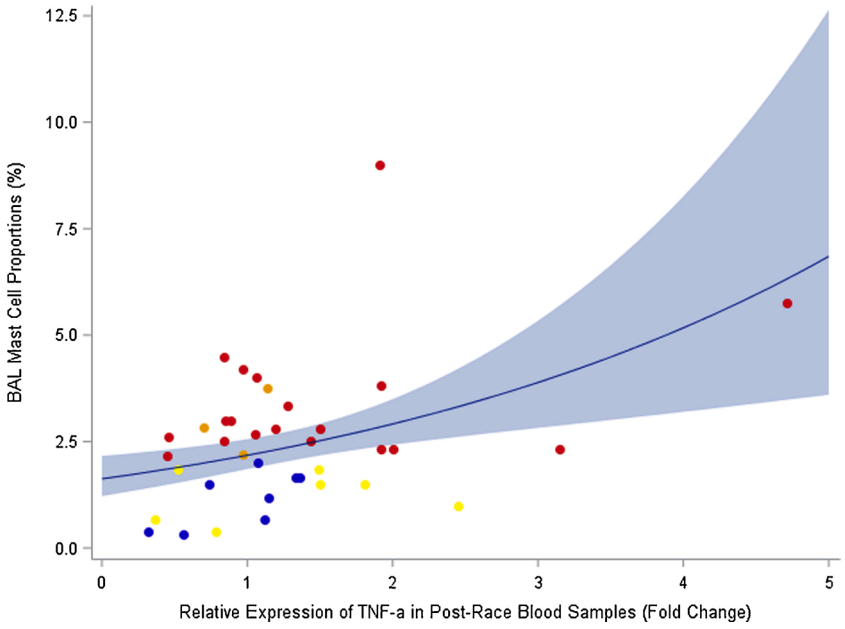

Fig. 5. Generalized linear mixed model of BAL mast cell proportions versus TNF-α gene expression in post-race peripheral blood samples. Blue: healthy horses; red: mast cell inflammation; orange: mixed inflammation; yellow: neutrophilic inflammation; solid line: predicted mean response; band: 95 % confidence interval of mean response; closed circles: observations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

gene expression in horses with MEA compared to healthy control horses, but horses with MEA in those studies had respiratory clinical signs or exercise intolerance (Beekman et al., 2012; Hughes et al., 2011; Lavoie et al., 2011). A separate study reported increased TNF-α concentration in BALF from horses with subclinical lower airway inflammation, but only horses with neutrophilic inflammation were included (Richard et al., 2014). Furthermore, previous studies have performed BALF collection at rest, while BALF was collected within 1 h of racing in our study; thus, it is possible that there was insufficient time to detect an increase in cytokine gene expression. This time point for post-race sample collection was selected because it is optimal for endoscopic detection and scoring of blood and mucus and avoids the confounding effect of intense exercise on BALF inflammatory cell proportions (Couetil et al., 2016; Couetil and Denicola, 1999; Hinchcliff et al., 2015).

To our knowledge, this is the first report documenting resting peripheral blood inflammatory cytokine gene expression in racehorses with lower airway inflammation. Several studies comparing peripheral blood cytokine gene expression or production in horses with severe equine asthma and in healthy control horses found no difference between groups (Horohov et al., 2005; Niedzwiedz et al., 2016; Vanderstock et al., 2018). Thus, results of the current study were not surprising given that horses with lower airway inflammation not associated with

clinical signs are not as severely affected as those with severe equine asthma.

Interleukin-8 gene expression was higher in post-race peripheral blood samples compared to resting peripheral blood samples, regardless of whether lower airway inflammation was present or not. This suggests that exercise alone increased IL-8 gene expression. Numerous studies have documented increases in inflammatory cytokine gene expression in response to strenuous exercise in human and equine athletes (Ainsworth et al., 2003; Cywinska et al., 2012; Lavin et al., 2020; Liburt et al., 2010; Nielsen et al., 2016; Suzuki et al., 2002). In racehorses, transient increases in peripheral blood inflammatory cytokine gene expression or production, including TNF-α, IL-1β, IL-6, and IL-8, are common and reflect systemic inflammation secondary to skeletal muscle inflammation (Donovan et al., 2007; Horohov et al., 2012; Liburt et al., 2010; Page et al., 2017). However, these increases in inflammatory markers are typically observed in unfit horses or those that have recently begun training (Liburt et al., 2010; Page et al., 2017), with inflammatory cytokine increases observed $>$ 2 h post-exercise (Donovan et al., 2007; Liburt et al., 2010). The horses in the current study were in active race training and were therefore less likely to exhibit systemic inflammation secondary to exercise. Furthermore, post-race peripheral blood samples were obtained within 1 h of race completion, so although it is unlikely that systemic inflammation was present, it is possible that inflammatory cytokines might have been increased at later time points. Endurance horses also demonstrate transient systemic inflammation that is characterized by increases in inflammatory cytokine gene expression during exercise, but unlike other cytokines, IL-8 gene expression typically remains elevated after ride completion (Cappelli et al., 2009; Page et al., 2019). Given that peripheral blood gene expression of TNF-α, IL-1β, and IL-6 remained unchanged post-race in the current study's population of horses, interpretation of increased IL-8 gene expression is difficult. It is possible that transient exercise-induced systemic inflammation was present but insufficient to induce expression of other inflammatory cytokines. Post-race peripheral blood IL-6 gene expression was lower in horses with mixed lower airway inflammation compared to other inflammatory phenotypes, but with only 3 horses in the mixed inflammation group, the clinical significance of this finding is unclear.

Horses with MEA typically demonstrate increases in gene expression of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-8 and IL-17 (Beekman et al., 2012; Hughes et al., 2011; Lavoie et al., 2011); however, this was not observed in the current study. These differences might be explained by the presence of subclinical lower airway inflammation in this horse population compared to other studies that included horses with respiratory clinical signs or exercise intolerance. Furthermore, the relationship observed in the current study between BALF neutrophil proportions and IL-1β relative gene expression suggests that differences in IL-1β expression would be expected in horses with more severe airway inflammation. In one study, horses in active training with lower airway inflammation not associated with clinical signs had increased TNF-α BALF concentrations compared to healthy control horses, but BALF sampling times were not specified beyond $\leq$ 1 month post-race, and can therefore not be directly compared to sampling times of 1 h post-race in the current study (Richard et al., 2014). Additionally, relative gene expression does not necessarily correlate directly with protein concentrations, especially across different disease states (Liu et al., 2016).

In this study, BALF mast cell proportions increased with increasing TNF-α gene expression in post-race peripheral blood, but not in resting peripheral blood or BALF. Mast cells are capable of synthesizing TNF-α which is then stored in mast cell granules. It is possible that exposure to allergens during exercise might have exacerbated production of TNF-α in allergen-primed BALF mast cells with subsequent release into circulation (Brightling et al., 2008). However, the lack of correlation between BALF mast cells and BALF TNF-α gene expression suggests that caution should be used when interpreting this data. It is possible that increased peripheral blood TNF-α gene expression was merely reflective of systemic inflammation, especially since the majority of horses with lower airway inflammation in this study were classified as mastocytic.

The main limitations of the current study included the small number of horses within each airway inflammation phenotype, the non-random sampling, and the lack of resting BALF samples. Inflammatory cytokine gene expression between resting and post-race BALF samples could not be compared as was done with peripheral blood. In addition, inflammatory cytokine gene expression might not reflect cytokine production, and cytokine concentrations in peripheral blood or BALF inflammatory cytokine were not evaluated.

In conclusion, the overall lack of inflammatory cytokine responses in BALF and peripheral blood in this population of racing Thoroughbreds might have been due to lower airway inflammation that was subclinical (i.e. did not cause respiratory clinical signs or exercise intolerance). Exercise alone increased peripheral blood gene expression of IL-8 in all horses, which might reflect mild systemic and airway inflammation. Investigation of a more comprehensive set of target genes in a larger group of horses is required to further define cytokine expression profiles among horses with different airway inflammation phenotypes.

Off-label antimicrobial use

N/A.

Institutional animal care and use committee approval

This study was approved by Purdue University's Institutional Animal Care and Use Committee.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Letters to the Editor

More on the crisis in veterinary medicine

Ever since I started my career as a poultry veterinarian and professor at the University of California-Davis School of Veterinary Medicine, I have worried about the training veterinary students receive in preventative veterinary medicine and herd and flock health.[1] The level of training in population medicine, particularly poultry, for veterinary students in the United States is typically so limited that students who track in small animal medicine often do not have any real exposure to flock or poultry medicine. At the same time, expertise at the university level is being reduced as faculty retire or the administration prioritizes other areas. In my mind, it is imperative to restructure the veterinary medicine curriculum to include preventative veterinary medicine aspects, including population medicine, since these aspects of veterinary medicine are critically important in controlling foreign animal diseases and maintaining food security and safety. A clear example of the lack of poultry medicine training is the poor reputation veterinarians have dealing with poultry cases and the fact that some groups raising poultry look for expertise abroad or through the internet. This topic deserves brainstorming and a clear strategy so that future veterinarians will be prepared to protect animal health and our food supply.

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African swine fever in wild pigs

I read with interest the recent *JAVMA* News article on African swine fever (ASF),[1] which noted that controlling this disease will require widespread cooperation. The article closed by noting the USDA had reached an agreement with the Canadian Food Inspection Agency on the steps to be taken following detection of ASF that, among other things, dealt with how affected countries should remove feral swine.

In Africa, ASF is maintained in a sylvatic transmission cycle involving warthogs (*Phacochoerus africanus*) and soft ticks (*Ornithodorus* spp). Over the past 3 decades, the geographic area of the United States that contains feral swine or European-type wild boars (*Sus scrofa*) has more than doubled, and in some areas, their numbers have been difficult or impossible to control. In addition, the United States is home to native species of *Ornithodorus* ticks, although their vector competence is unknown.[2] The susceptibility of peccary or javelina (*Tayassu tajacu*) populations is also unknown. It is critical that swine veterinarians in the United States and Canada prepare for ASF, but it is not clear what preparations are being made to deal with ASF in wild pigs scattered across the wildland portions of 27 states, 3 territories, and 2 provinces when, not if, the disease arrives here. Removal of wild swine does not seem to be a viable option.

The ASF virus is highly infectious and lasts for a considerable period of time (months to years) in urine, feces, blood, carcasses, soils, and prepared and frozen meats; on knives, boots, tires, and other surfaces[3]; and even in unprocessed bulk-shipped grain.[1] When the virus is introduced into an ASF-free wild boar population, epidemics almost always occur, which may lead to a decrease in the wild boar population[3] and fade-out of the disease, but reappearance within months is common, likely as a result of wild boars moving within an infected area and contacting the virus in wild boar carcasses.[3] Although the virus tends to remain endemic in previously infected areas, it also spreads by movement and contact into unaffected neighboring wild boar groups.[3]

In some states and provinces, wild pigs are game animals under the jurisdiction of the state or provincial wildlife agency. In others, they are under the jurisdiction of the agriculture agency, and in some, they are considered alternative livestock. Extensive populations of wild swine now live on private properties and on lands under the authority of a variety of federal agencies including, but not limited to, the National Park Service, US Forest Service, Bureau of Land Management, and Department of Defense. Lack of access or infrastructure, differing policies

Instructions for Writing a Letter to the Editor

Readers are invited to submit letters to the editor. Letters may not exceed 500 words and 6 references. Letters to the Editor must be original and cannot have been published or submitted for publication elsewhere. Not all letters are published; all letters accepted for publication are subject to editing. Those pertaining to anything published in the *JAVMA* should be received within 1 month of the date of publication. Submission via email (*JournalLetters@avma.org*) or fax (847-925-9329) is encouraged; authors should give their full contact information, including address, daytime telephone number, fax number, and email address.

Letters containing defamatory, libelous, or malicious statements will not be published, nor will letters representing attacks on or attempts to demean veterinary societies or their committees or agencies. Viewpoints expressed in published letters are those of the letter writers and do not necessarily represent the opinions or policies of the AVMA.

and priorities, and lawsuits may hinder ASF surveillance and control, even during a declared disease emergency. Aggressive hunting in Texas, including helicopter pursuit and the use of automatic rifles, harvests only about 5% of wild pigs yearly, well below their reproductive capacity. Vaccination may prove useful for the swine industry[1] but seems unlikely to be effective in wild swine distributed across vast landscapes.

African swine fever is a disease at the wildlife-livestock interface where wildlife are critical potential reservoirs of persistent infection, and any response or control effort must effectively deal with them and the ecology of the disease. With the likelihood that ASF will eventually arrive in North America, perhaps it is time for federal government agencies, swine veterinarians, and state agriculture agencies to start working with wildlife veterinarians, conservationists, and wildlife agencies.

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First reported detection of the equine herpesvirus 1 DNA polymerase 2254C/His752 variant in horses in the United States

Equine herpesvirus 1 (EHV1) is an important viral pathogen of equids that can cause substantial economic losses. Infection causes a spectrum of disease manifestations in horses, including respiratory disease, abortion, neonatal death, and myeloencephalopathy. Although both host and viral factors are important for EHV-1 infection, a single-nucleotide variation within open reading frame 30 (ORF30) that encodes for the viral DNA polymerase (ie, an A-to-G substitution at nucleotide 2254 resulting in replacement of asparagine at residue 752 with aspartic acid) has been shown to be strongly (76% to 86%) associated with neuropathogenicity.[1,2]

A new EHV-1 variant (strain FR-56628) with cytosine at nucleotide 2254 in ORF30, resulting in an amino acid change to histidine at residue 752, was recently described in France.[3] We report here detection of this new EHV-1 variant in samples from 2 horses in the United States, unrelated to the French outbreak, that were submitted to the Pennsylvania Animal Diagnostic Laboratory System in March 2021. The first horse was admitted to the University of Pennsylvania School of Veterinary Medicine large animal hospital at the New Bolton Center with signs consistent with equine herpes myeloencephalopathy (EHM) and was euthanized. Genetic epidemiological studies are ongoing. The second horse was identified around the same time. This horse was febrile, had neurologic signs, and was also euthanized. It had not been at the New Bolton Center but had been in contact with a horse that had been briefly hospitalized during the risk period. Both of these horses were positive for EHV-1 with a real-time PCR assay targeting the glycoprotein B gene (Ct values of 32.78 and 25) but were negative with an allelic-discrimination (2254A>G) real-time PCR assay.[4] Consequently, we amplified a 256–base pair fragment of ORF30 flanking nucleotide position 2254,[5] and sequences from both horses had 100% identity with the ORF30 sequence for the FR-56628 EHV-1 strain, confirming the presence of cytosine at nucleotide 2254. Because of this identified variation, veterinarians submitting samples to establish a diagnosis of EHV-1 infection should request the use of a PCR assay that targets consensus regions (eg, the glycoprotein B gene) rather than the use of a neuropathogenic strain–typing assay.

Although this viral variant has been detected in multiple other horses epidemiologically linked to the 2 horses with EHM, exposed and infected horses have not always developed clinical signs or died, and most related confirmed cases have had the usual array of clinical signs typical of EHV-1 infection, including pyrexia, vasculitis, edema, and, in some cases, ataxia. However, it could be noteworthy that in the 2 horses euthanized because of EHM, the first clinically apparent sign was hind limb edema, which was noted ≥ 2 days prior to the development of neurologic signs.

To our knowledge, this represents the first reported detection of the EHV-1 2254C genotype in horses unrelated to the reported French outbreak. Further studies are warranted to understand prevalence of this genotype and its impact on the equine industry in North America.

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Emerging outbreak of hepatitis in Midwestern horses

We are writing to inform *JAVMA* readers of a potential emerging outbreak of hepatitis in horses. Recognition of cases in several Midwestern states, including Indiana, Illinois, Kentucky, and Michigan, began in the autumn of 2020 and continues to date. Additionally, a few horses with similar signs have been reported in other areas of the country.

The hallmarks of disease include high fever (39.4°C to 41.7°C [103°F to 107°F]) that may be biphasic or triphasic with 7- to 10-day periods of normothermia between fever cycles. Horses are lethargic and anorectic while febrile, but no localizing signs are present. Affected horses vary in age, breed, and sex, and affected broodmares have given birth to healthy foals. Serum activities of γ-glutamyltransferase and sorbitol dehydrogenase are consistently high, and high aspartate aminotransferase and alkaline phosphatase activities have been documented in some horses. Unconjugated serum bilirubin concentrations are typically high, and serum amyloid A concentration is consistently high (500 to 1,000 mg/dL). Neutrophilia is the most common cytologic abnormality. Toxic neutrophils are present in some horses when initial clinical signs appear. Histologic examination of liver biopsy samples has revealed acute cholangiohepatitis with infiltrations of neutrophils, lymphocytes, and macrophages in some cases and portal hepatitis in others. In some samples obtained during the acute phase of the disease, islands of necrosis are present. At this time, an etiology has not been identified, and results of the following diagnostic tests have been negative in affected horses:

- Fecal culture for *Salmonella* spp.
- Anaerobic fecal culture for *Clostridium* spp.
- Fecal PCR assay for *Neorickettsia risticii*, *Clostridioides difficile* toxin genes, *Lawsonia intracellularis*, *Salmonella* spp, and coronavirus.
- Aerobic and anaerobic microbial culture of liver tissue.
- Electron microscopic examination of liver tissue.
- Virus isolation from liver tissue.
- Serum and liver PCR assay for equine parvovirus-hepatitis and nonprimate hepacivirus (ie, equine hepacivirus) in horses from Indiana (up to 70% of affected horses and 30% of unaffected pasture mates from Kentucky have been positive for equine hepacivirus in serum samples).
- Whole blood PCR assay for *Anaplasma phagocytophilum*.
- Urine PCR assay and serum antibody titers for *Leptospira* spp.
- Nasal swab PCR assay for equine herpesvirus 1 and 4, equine influenza A, *Streptococcus equi* subsp *equi*, equine rhinitis A virus, and equine rhinitis B virus.
- Nasal swab, nasopharyngeal swab, and fecal assays for SARS-CoV-2.

The high fevers observed in affected horses typically respond to NSAIDs. It is unclear whether antimicrobial treatment is necessary. In some cases, antimicrobials appeared to have no effect on preventing a second or third cycle of fever; in others, long-term (3 to 4 weeks) antimicrobial treatment has been associated with the prevention of additional fever cycles and normalization of hepatic enzyme activities. Most affected horses make a full clinical recovery with hepatic enzyme activities decreasing over 4 to 12 weeks. However, it is unknown whether hepatic fibrosis will occur in the future as a consequence of hepatitis.

Readers are encouraged to contact Dr. Sandra Taylor if they have suspect cases or questions.

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APPENDIX E

Refereed Scientific Publications:

- Anis, E., Ilha, M.R.S., Engiles, J.B., Wilkes, R.P**. Evaluation of Targeted Next-Generation Sequencing for Detection of Equine Pathogens in Clinical Samples.** *Journal of Veterinary Diagnostic Investigation*, in press, 2020.

- Hay, A.N., Wagne,r B., Leeth, C.M., LeRoith, T., Cecere, T.E., Lahmers, K.K., Andrews, F.M., Were, S.R., Johnson, A.L., Clark, C.K., Pusterla, N., Reed, S.M., Lindsay, D.S., Taylor, S.D., Estell, K.E., Furr, M., MacKay, R.J., Del Piero F., *Witonsky, S.G**. Horses Affected by EPM have Increased sCD14 Compared to Healthy Horses.** *J Immunol Immunopathol*. 2021;242, DOI: 10.1016/j.vetimm.2021.110338.

- Hohu, K.K., Lim, C.K., Adams, S.B., Heng, H.G., Ramos-Vara, J.A. **Ultrasonographic and Computed Tomographic Features of Rice Bodies in an Arabian Horse with Atlantal Bursitis.** Vet Radiol Ultrasound. 2020 Jan;61(1):E1-E5. doi: 10.1111/vru.12596.

- Keenan, A.V., Townsend, W.M. **Evaluation of Equine Corneal Disease using Ultrasound Biomicroscopy.** *Vet Ophthalmol.* 2021;00:1-6. Published online 11 March 2021. https://doi.org/10.1111/vop.12881.

- Lyons, V.N., Townsend, W.M., Moore, G.E., Liang, S. **Commercial Amniotic Membrane Extract for Treatment of Corneal Ulcers in Adult Horses.** *Equine Vet J.* 2021;00:1-9. Dec 15. doi: 10.1111/evj.13399.

- Skelton, J.A., Hawkins, .J.F, .Rochat, M.C. **Treatment of Shoulder Joint Luxation with Glenoid Ostectomy in a Miniature Donkey.** *J Am Vet Med Assoc*. 2021 Nov 1;259(9):1043-1046. doi: 10.2460/javma.259.9.1043.

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Evaluation of targeted next-generation sequencing for detection of equine pathogens in clinical samples

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Abstract. Equine infectious disease outbreaks may have profound economic impact, resulting in losses of millions of dollars of revenue as a result of horse loss, quarantine, and cancelled events. Early and accurate diagnosis is essential to limit the spread of infectious diseases. However, laboratory detection of infectious agents, especially the simultaneous detection of multiple agents, can be challenging to the clinician and diagnostic laboratory. Next-generation sequencing (NGS), which allows millions of DNA templates to be sequenced simultaneously in a single reaction, is an ideal technology for comprehensive testing. We conducted a proof-of-concept study of targeted NGS to detect 62 common equine bacterial, viral, and parasitic pathogens in clinical samples. We designed 264 primers and constructed a bioinformatics tool for the detection of targeted pathogens. The designed primers were able to specifically detect the intended pathogens. Results of testing 27 clinical samples with our targeted NGS assay compared with results of routine tests (assessed as a group) yielded positive percent agreement of 81% and negative percent agreement of 83%, overall agreement of 81%, and kappa of 0.56 (moderate agreement). This moderate agreement was likely the result of low sensitivity of some primers. However, our NGS assay successfully detected multiple pathogens in the clinical samples, including some pathogens missed by routine techniques.

Key words: equine; infectious diseases; next-generation sequencing.

Introduction

The laboratory detection of infectious disease agents, especially in the case of mixed infections with 2 or more pathogens, is challenging to the clinician and the diagnostic laboratory. Routinely, submission is required of multiple samples to separate laboratory sections. Clinical microbiology has traditionally relied on isolation of pathogens by culture, followed by biochemical and other tests to identify the genus or species. Also, molecular testing, including PCR, has been used widely for detection of many viral and bacterial pathogens.[7,10,23] However, because most of these tests detect only one or a few pathogens, multiple tests are often required to identify the potential causative agents, especially when clinical signs are nonspecific. Next-generation sequencing (NGS) can overcome this limitation by simultaneously testing for numerous infectious agents in a single tube. Therefore, NGS-based testing is being adopted in diagnostic microbiology.[3,9,21]

NGS studies of microorganisms typically follow 1 of 2 strategies: whole-genome sequencing (WGS) or targeted sequencing.[24,28] WGS has the potential to sequence all nucleic acids within a sample and provides complete characterization of the genomic content. However, this unbiased sequencing requires a substantial amount of sequence depth, particularly for viruses, to separate low-prevalence pathogens from the overwhelming contribution of host nucleic acid.[17] In contrast, targeted sequencing uses target-specific primers for PCR-mediated amplification, such that the genomic regions of interest are enriched and selectively sequenced. Compared to unbiased sequencing, targeted sequencing provides better coverage, specificity, and ease of downstream analysis.[2,12,14,20] Hence, targeted NGS is a promising tool to provide comprehensive assays for the detection of known, clinically relevant pathogens from a variety of specimens, particularly for cases with nonspecific disease indications that may be associated with multiple infectious agents. Studies have demonstrated the feasibility of using targeted sequencing for clinical infectious disease testing.[1,15,30] Further, combining targeted sequencing with WGS can significantly improve the recovery of whole

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genome sequences of viruses (e.g., Hantaan orthohantavirus[18]) directly from clinical samples.

We evaluated the use of targeted NGS for detection of 62 equine pathogens in clinical samples. We tested 27 clinical equine samples with the targeted NGS assay as well as with routine laboratory methods to evaluate the feasibility of applying a targeted NGS technique in a clinical molecular laboratory for both testing of clinical submissions and disease surveillance.

Materials and methods

Design of amplicon panel primers

Primers were designed for 62 equine pathogens selected for their relevance to clinical equine disease observed in the field, including bacterial, fungal, viral, and parasitic pathogens (Suppl. Table 1). The primers were designed to target specific regions of each pathogen (~200 bases per target region) based on suitable regions published in the literature, using the AmpliSeq Designer (Ion Torrent; Thermo Fisher). Based on in silico analysis of primer specificity using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), changes were made to the design with the assistance of the AgriSeq bioinformatics team (Ion Torrent; Thermo Fisher). Because variation in targeted organisms could cause individual primers to fail, multiple primers per targeted organisms were designed to provide redundancy. Based on the assay design, the primers were separated into 2 primer pools to reduce binding between the primers.

Nucleic acid extraction, library preparation, and NGS

Total nucleic acid (both DNA and RNA) was isolated (DNeasy blood and tissue kit; Qiagen). A modification of the animal tissue protocol was employed as described previously.[1]

Automated library preparation, template preparation, and chip loading were performed (Ion Chef instrument; Thermo Fisher) as described elsewhere.[1] Briefly, reverse-transcription PCR (RT-PCR) using the designed primer pools and library preparation were performed (Ion Chef, AmpliSeq kit for Chef DL8; Thermo Fisher), according to the manufacturer's protocol. This kit allowed the preparation of 8 barcoded Ion AmpliSeq libraries per Ion Chef run (8 different clinical cases). Then, 50 pmol of the 8 mixed libraries were used to prepare the template and load a chip (Ion 314 chip, Ion Chef instrument, Ion PGM kit; Thermo Fisher), according to the manufacturer's instructions. Finally, the libraries were sequenced (Ion PGM Hi-Q View sequencing kit, Ion Torrent personal genome machine; Thermo Fisher), according to the manufacturer's instructions.

Automated preparation minimizes sample handling and lowers chance of contamination as well as provides reproducible chip loading. This automated workflow generated results within 2–3 d.

Data analysis

A reference file containing the sequences of the targeted pathogens obtained from GenBank was constructed and uploaded to the Ion Torrent suite software (Thermo Fisher). These files were used for initial data analysis with the Torrent suite software, including read trimming, assembly with SPAdes (http://coolgenes.cahe.wsu.edu/ion-docs/Assembler-SPAdes-Plugin.html), and mapping to the reference file. Then, the Bam files were downloaded and evaluated with Geneious software (v.9.1.2; Biomatters). Finally, pathogen identifications were confirmed with BLAST. BLAST (E) values <1 were considered acceptable. The lower the E-value, or the closer it is to zero, the more "significant" the match of the alignments (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=FAQ#expect).

Assay analytical performance

Both analytical sensitivity (ASe; relative limit of detection [RLOD]) and analytical specificity (ASp) were determined to assess the analytical performance of the assay. The relative ASe of a subset of the organisms was assessed for proof-of-concept testing of the design for detection of these pathogens in clinical samples. This was performed by testing relatively known quantities (based on quantitative PCR [qPCR] or reverse-transcription real-time PCR (RT-rtPCR) results, cycle threshold [Ct] values) of DNA from representatives of the viral, bacterial, fungal, and parasitic pathogen groups, as well as viral RNA. Seven clinical samples containing very low levels (Ct 30–35) of organisms, including *Clostridioides difficile*, *Clostridium perfringens*, *Salmonella* spp., *Streptococcus equi* subsp. *equi*, *Neorickettsia risticii*, equine herpesvirus 1 (EHV-1), and West Nile virus (WNV), were tested to determine the RLOD. Typically, for qPCR or RT-rtPCR testing, higher Ct values (>35) are considered suspect because they may only represent fluorescence artifacts or cross-contamination.[6] The qPCR or RT-rtPCR assays were performed at the Tifton Veterinary Diagnostic and Investigational Laboratory, College of Veterinary Medicine, University of Georgia (TVDIL; Tifton, GA), Indiana Animal Disease Diagnostic laboratory, College of Veterinary Medicine, University of Purdue (ADDL; West Lafayette, IN), and Veterinary Diagnostic Laboratory, University of Kentucky (UKVDL; Lexington, KY) with laboratory-validated procedures. These laboratories are accredited by the American Association of Veterinary Laboratory Diagnosticians (requirements are based upon the ISO/IEC 17025:2005 standard, https://www.iso.org/standard/39883.html).

ASp was performed to determine the ability of the assay to detect the targeted pathogens without being affected by

specimen-related conditions or cross-reactivity and/or interference of the host nucleic acid. To assess the detection ability of the assay and to better mimic clinical samples, a sample known to contain 1 pathogen based on previous testing was spiked with equal amounts of 4 other pathogens. The assay was evaluated by testing validated isolates or reference strains of most of the pathogens (bacteria, parasites, fungi, and viruses) that can be detected using this panel (Table 1) Eight pathogens were not tested, namely *Clostridium tetani*, *Taylorella equigenitalis*, *Babesia caballi*, *Theileria equi*, *Leishmania* sp., *Neospora hughesi*, *Trypanosoma cruzi*, *Halicephalobus* sp., *Campylobacter coli*, Venezuelan equine encephalitis virus (VEEV), equine coronavirus (ECoV), and equine adenovirus 1 and 2.

Assay clinical evaluation and statistical analysis

Determination of the diagnostic sensitivity and specificity of an assay requires the evaluation of every detected pathogen in every clinical case and comparing the sensitivity and specificity with that of gold standard test results for known positive and negative samples. Instead, we compared the NGS panel with routine laboratory methods for detection of these pathogens in clinical samples for proof-of-concept testing, considering that the purpose of the NGS test would be to replace these routine methods. We determined the positive percent agreement (PPA), negative percent agreement (NPA), and total agreement between the NGS method and the routine methods used collectively for case diagnosis. We included in the comparative study 27 equine clinical cases that were submitted to TVDIL, ADDL, UKVDL, and Pennsylvania Animal Diagnostic Laboratory System–New Bolton Center, School of Veterinary Medicine, University of Pennsylvania (PADLS-NBC; Kennett Square, PA) in 2018–2019. We evaluated a broad range of sample types and clinical conditions for our proof-of-concept testing. Samples had been sent to each laboratory for diagnostic purposes, and not all samples were submitted with complete histories.

Each case was tested via the designed targeted NGS assay as well as with routine laboratory methods. The routine tests included bacterial culture, ELISA, PCR, RT-rtPCR, and qPCR. Each of the routine tests had been done by the submitter laboratory following their laboratory-validated procedures. The relative PPA and NPA were assessed with respect to routine methods as a group.[11] Also, the overall agreement and Cohen kappa were assessed by comparing the new assay to routine methods as a group (if any of a group of agents was not detected by the new assay or routine methods, the result for the test group was considered negative). Cohen kappa is the standard agreement coefficient that considers the possibility of the agreement occurring by chance. Cohen kappa is always ≤1; a value of 1 indicates perfect agreement between 2 tests, and 0 indicates that any agreement is the result of chance.[16]

Results

Our newly designed primer pools were able to specifically detect and/or sequence the target region of all bacteria, fungi, parasites, and viruses that we tested. For the LOD, 7 representative bacteria, viral DNA, and viral RNA were tested. The targeted equine NGS panel was able to detect pathogens with Ct values of 30–35 for 5 of the tested targets. Primer sets that targeted *N. risticii* and *C. difficile* resulted in poor sequencing coverage (low number of reads) or no sequence, respectively, when tested with clinical samples that were positive by qPCR (Ct = 29.5).

We evaluated the targeted NGS method using 27 equine clinical samples representing neurologic (n = 2), abortion (n = 3), intestinal (n = 12), and respiratory (n = 9) diseases, and a cutaneous wound (n = 1), all tested previously using other routine methods. Targeted NGS not only successfully identified multiple pathogens in the clinical samples in concordance with routine methods but also identified pathogens not detected by the routine techniques (Table 2). Specifically, targeted NGS detected EHV-1 in 1 of 2 neurologic and in 3 of 3 abortion cases that were positive via qPCR with Ct values of 15–32.9. In the second neurologic case, WNV was detected by both targeted NGS as well as RT-rtPCR, with good correlation with pathology findings. Of 12 intestinal samples, there was agreement between targeted NGS and routine microbiologic techniques in 8 of 12 cases, with good correlation with pathology findings in 6 of 11 samples for which pathology results were available. Cases in which targeted NGS and routine microbiologic results were not correlated included one fecal sample for which targeted NGS failed to detect any pathogens but in which rotavirus, *C. difficile*, and *C. perfringens* were detected by RT-rtPCR and qPCR with Ct values of 37.7, 37.1, and 37.1, respectively. Interestingly, pathology findings included mycotic pneumonia and cecocolonic hyperemia without evidence of overt enterocolitis, making interpretation of the clinical significance of these PCR findings difficult. The other intestinal cases without agreement between microbiologic testing methods and NGS included 2 cases in which pathogens in intestinal contents were only identified using targeted NGS. In one case, *Actinobacillus* sp. was identified in intestinal contents from a horse with enteritis confirmed by pathology; in another case, *Pseudomonas aeruginosa* septicemia was diagnosed by splenic culture and later confirmed by pathology.

Among the 9 samples from respiratory cases, comprising swabs, fluid from transtracheal washes, and lung tissue, only 3 of 9 had pathology results available for comparison, but they had good correlation between lesions and pathogens identified by both testing methods. However, our targeted NGS failed to amplify EHV-5 in 1 case (Ct 37.0) without available pathology results, and failed to amplify EHV-2 in 2 cases (Ct 37.0 and 39.8, respectively); in 1 of these 2 cases, EHV-5 was detected by both methods. In one case of a

Table 1. List and source of isolates and reference strains of the pathogens that can be detected by the targeted NGS assay.

Pathogen	Source*
Eastern equine encephalitis virus	B
Equine rhinitis A virus	A
Equine rhinitis B virus	A
Equine arteritis virus	A
Equine herpesvirus1	A
Equine herpesvirus 2	A
Equine herpesvirus 3	A
Equine herpesvirus 4	A
Equine herpesvirus 5	B
Equine influenza A virus, Miami (H3N8)	A
Equine influenza A virus, Prague (H7N7)	A
Rotavirus A, Nebraska strain	A
Saint Louis encephalitis virus	B
Western equine encephalitis virus	B
West Nile virus	B
Vesicular stomatitis virus, Indiana strain	B
Vesicular stomatitis virus, New Jersey strain	B
Clostridioides difficile strains that possess A, B, and binary toxin genes	B
Clostridium perfringens strains that possess alpha (α), beta (β), beta-2 (β-2) enterotoxin (CPE), epsilon (ε), iota (ι) toxin genes	B
E. coli strains that possess Shiga toxin 1 (stx1), intimin (eae), alpha hemolysin (hlyA), cytotoxic necrotizing factor (cnf1 and cnf2), enterotoxin (STa), fimbrial (k99), and F41 virulence factor genes	C
Actinobacillus sp. (*A. pleuropneumoniae* tested)	B
Anaplasma phagocytophilum	D
Aspergillus spp. (*A. fumigatus* tested)	B
Bordetella bronchiseptica	B
Borrelia burgdorferi	B
Chlamydia sp. (*C. felis* tested)	B
Cryptococcus spp. (*C. neoformans* tested)	C
Dermatophilus congolensis	B
Giardia intestinalis	C
Histoplasma capsulatum	B
Klebsiella sp. (*K. pneumoniae* tested)	B
Lawsonia intracellularis	B
Leptospira spp. (serovar Pomona tested)	B
Listeria monocytogenes	B
Mycoplasma sp. (*M. bovis* tested)	B
Neorickettsia risticii	E
Neospora caninum	F
Nocardia sp. (*N. nova* tested)	B
Pneumocystis carinii	B
Prototheca spp. (*P. zopfii* tested)	H
Pseudomonas sp. (*P. aeruginosa* tested)	B
Pythium spp. (*P. insidiosum* tested)	B
Rhodococcus equi	B
Salmonella spp.	B
Sarcocystis neurona	G
Sporothrix spp. (*S. schenckii* tested)	B
Staphylococcus aureus	B
Streptococcus equi subsp. *equi* (*S. equi*)	B
Streptococcus sp. (*S. dysgalactiae* tested)	B
Toxoplasma gondii	F

* A = USDA National Veterinary Services Laboratories; B = detected in a diagnostic sample and identified and isolated in the TVDIL according to a validated protocol as follows: bacterial isolates were identified via biochemical testing and a commercial microtiter system; viral isolates were identified via fluorescent antibody test or RT-qPCR; C = *E. coli* Reference Center, Pennsylvania State College of Agriculture Sciences, University Park, PA; D = detected in a diagnostic sample and identified via specific qPCR by the Clinical Immunology and Virology laboratory, College of Veterinary Medicine, University of Tennessee (Knoxville, TN); E = detected in a diagnostic sample by specific qPCR by the Indiana Animal Disease Diagnostic laboratory, Purdue College of Veterinary Medicine, University of Purdue (West Lafayette, IN); F = validated reference kindly provided by Drs. Chunlei Su and Rick Gerhold, University of Tennessee; G = validated reference kindly provided by Dr. Daniel K. Howe, University of Kentucky (Lexington, KY); H = validated reference kindly provided by Dr. Amy Swinford, Texas A&M Veterinary Medical Diagnostic Laboratory (College Station, TX).

Table 2. Clinical case comparative study results of the targeted NGS assay and the routine methods.

		Laboratory findings	
Case/Specimen	Autopsy finding or diagnosis	Targeted NGS	Routine methods (PCR, bacterial culture, or ELISA)
1–Spinal cord	Myeloencephalopathy, chronic bacterial cyst. Multifocal hepatic necrosis.	EHV-1.	EHV1 (Ct 32.9).
2–Pooled cerebrum, brainstem, and spinal cord	WNV encephalomyelitis.	WNV; EHV-2; EHV-5 (few reads).	WNV (Ct 25.2). Negative for EEEV and EHV-1.
3–Fetal tissue pool	Equine herpesviral abortion.	EHV-1.	EHV-1 (Ct 17). Negative for *Leptospira* sp. by PCR.
4–Fetal tissue pool	Equine herpesviral systemic disease; perinatal death.	EHV-1.	EHV-1 (Ct 20).
5–Fetal tissues pool	Equine herpesviral abortion.	EHV-1.	EHV-1 (Ct 15).
6–Feces	Not available.	*N. risticii* (few reads).	*N. risticii* (Ct 29.23).
7–Feces	Acute necrotizing enterocolitis.	*C. difficile* A and B toxin genes. *C. perfringens* alpha toxin gene. *Salmonella* sp., *E. coli*, adenovirus-1.	*C. difficile* A and B toxin genes Ct 29.8). *C. perfringens* (Ct 29.9). *E. coli* and *C. difficile* on colon culture. Negative for coronavirus, rotavirus, *L. intracellularis, N. risticii, Salmonella* sp.
8–Pooled duodenum/ ileum scraping	Duodenal perforation, peritonitis. Chronic eosinophilic enterocolitis.	*C. perfringens* CPE and alpha toxins genes.	Negative for *L. intracellularis* and *Salmonella* sp.
9–Feces	Mesenteric pyogranuloma. Mild histiocytic and neutrophilic hepatitis, myocarditis, interstitial nephritis; pyogranulomatous pneumonia.	No sequences detected.	Negative for rotavirus and coronavirus.
10–Pooled ileum/ colon scraping	Mycotic pneumonia. Cecocolonic mucosal hyperemia.	No sequences detected.	Rotavirus (Ct 37.7), *C. difficile* (Ct 37.1), *C. perfringens* (Ct 37.2). Negative for *L. intracellularis, Salmonella* sp., *N. risticii*, coronavirus.
11–Pooled small intestine/large intestine scraping	Moderate locally extensive erosive colitis. Moderate to marked vascular congestion is present in all layers of the small intestine. Neonatal encephalopathy.	*C. perfringens* alpha and beta toxins (few reads).	*C. difficile* A and B toxins positive via ELISA and PCR (Ct 29.6). *C. difficile* and *C. perfringens* were not isolated on colon and/or small intestine culture. Negative for coronavirus, rotavirus, *L. intracellularis, Salmonella* sp., *N. risticii, C. perfringens.*
12–Intestine scraping	Moderate, multifocal to coalescing, gastric ulcers. Necrotizing enterocolitis.	*C. perfringens* alpha toxin gene, *E. coli* Hly virulence factor.	*C. perfringens* (Ct 20.3).
13–Intestine scraping	Marked necrohemorrhagic enterocolitis with mild intestinal nematodiasis and mesenteric lymph node hyperplasia.	*C. perfringens* alpha toxin gene (few reads). *E. coli* CNF-1 and eae; *Cryptococcus* (few reads).	*C. perfringens* (Ct 34.7). Negative for *C. difficile, L. intracellularis, Salmonella* sp.
14–Intestinal contents	Clostridial enterocolitis.	*C. perfringens* alpha, beta, netF2, beta 2, and CPE toxin genes.	*C. perfringens* genotype A (Ct 15.2), *C. difficile* (Ct 36.5). *C. difficile* was not isolated from colon and/or small intestine tissues.
15–Intestinal contents	Bacterial colitis.	No sequences detected.	Negative for *C. difficile, C. perfringens, Salmonella* sp., *N. risticii*
16–Intestinal contents	Enteritis.	*Actinobacillus* sp.	Negative for *Salmonella* sp.

(continued)

Table 2. (continued)

Case/Specimen	Autopsy finding or diagnosis	Laboratory findings: Targeted NGS	Laboratory findings: Routine methods (PCR, bacterial culture, or ELISA)
17–Intestinal contents	*Pseudomonas aeruginosa* septicemia.	*P. aeruginosa, Streptococcus* sp.	*N. risticii* (Ct 30.8).
	Ulcerative duodenitis.		*C. perfringens* isolated on small intestine culture.
	Fibrinonecrotic typhlocolitis.		*P. aeruginosa* isolated on spleen culture.
18–Respiratory swab	Not available.	*S. equi*, EHV-2.	*S. equi* (Ct 32). Not tested for other pathogens.
19–Respiratory swab	Not available.	No sequences detected.	EHV-5 (Ct 37.0). Negative for EHV-2.
20–Respiratory wash	Not available.	*Pseudomonas* sp., *K. pneumoniae.*	Negative for EHV-5.
21–Respiratory swab	Not available.	No sequences detected.	Negative for EHV-2, EHV-5.
22–Respiratory swab	Not available.	EHV-5.	EHV-5 (Ct 25.1), EHV-2 (Ct 39.8).
23–Lung tissue from 50-d-old foal	Severe bronchointerstitial pneumonia with pyogranulomas and hyaline membranes.	*R. equi, P. aeruginosa*, EHV-2.	*R. equi, S. equi* subsp. *zooepidemicus*.
24–Respiratory swab	Not available.	*P. aeruginosa.*	EHV-5 (Ct 35.7), EHV-2 (Ct 37.0).
25–Lung tissue from 60-d-old foal	Bronchopneumonia.	*R. equi*, EHV-2.	*R. equi* isolated on lung culture.
26–Lung tissue	Necrotizing bronchopneumonia.	*S. aureus.*	*S. aureus* isolated on lung culture.
27–Wound swab	Not available.	*S. aureus, Streptococcus* sp., *Actinobacillus* sp.	*S. aureus, Streptococcus dysgalactiae* subsp. *equisimilis, E. coli.*

Table 3. Comparison of the results of the clinical cases tested by targeted NGS assay and routine methods by comparing the pathogens as a group.

Targeted NGS panel result	Routine method results: Positive	Negative	Total
Positive	17	1	18
Negative	4	5	9
Total	21	6	27

wound, there was good correlation between targeted NGS and routine bacterial culture.

Based on our comparative study, the overall agreement between our new assay and the routine methods used was 81%, with $\kappa = 0.56$ (moderate agreement). The PPA and NPA of the targeted NGS were 81% and 83%, respectively (Table 3).

Discussion

We evaluated the use of targeted NGS to detect equine pathogens directly from clinical samples. Our new assay was able to detect pathogens (*Clostridium perfringens*, *Salmonella* spp., *Streptococcus equi* subsp. *equi*, EHV-1, and WNV) that had high Ct values (29–32.9) when tested by qPCR, demonstrating the ability of the assay to detect some pathogens even at low levels. Obtaining good sequence recovery directly from clinical samples, particularly in samples with low levels of organism, has been a challenge for the application of NGS technology as a detection tool. However, the use of targeted NGS to generate a large number of sequences for the target gene or region significantly improves the detection of targets in degraded and low-titer samples.[1,8,29] Additionally, despite the limited testing performed, we were able to successfully detect pathogens with the targeted NGS method from multiple sample types.

Evaluation of ASe is usually done by testing the LOD with plasmids or in vitro transcribed RNA per target. Considering the lack of availability of all needed reference material to perform this type of testing and the prohibitive cost, we decided to limit this evaluation to RLOD for group representatives. These samples represent the different types of pathogens targeted by the protocol (bacterial DNA, viral DNA, and viral RNA) and were known from previous testing to contain very low levels of organisms (qPCR and RT-rtPCR Ct of 30–35). Although this may not be the recommended way to evaluate the LOD of an assay, it provided an estimate of the RLOD for the NGS design. Our targeted NGS equine assay is intended to detect organisms associated with clinical disease, therefore determining the absolute ASe was considered less important than if this test was intended to test for carrier status or for freedom from disease, for which a very low LOD is needed. Further evaluation of the LOD will be needed before the use of our assay as a routine test.

Not all pathogens for which primers were included in our panel were tested. Included in this group were the foreign animal disease pathogens. Primers for these pathogens were included in our assay to develop a comprehensive test but also as a means of providing expanded surveillance, which could be done along with routine testing with this type of assay. A justification for including some of these primers is the fact that climate change is allowing expansion of pathogens into new areas. It is important to note, however, that if a NGS assay detected a foreign animal disease pathogen, the proper authorities would need to be notified and appropriate confirmatory testing performed.

The overall agreement between the new assay and the routine methods was 81%. The main contributor to the reduced agreement was failure of the NGS method to detect very low levels of some organisms, which were detectable in the samples by qPCR. Primers for organisms that had reduced sensitivity based on this testing were those for *N. risticii*, *C. difficile*, EHV-2, and EHV-5. Samples with low amounts of these organisms had poor sequencing coverage (low number of reads) or no sequence.

For respiratory cases evaluated in our study, the significance of the qPCR results with Ct values in the high 30s for EHV-2 and EHV-5 in upper respiratory tract swabs is unknown. Both EHV-2 and EHV-5 produce persistent infections in the host. EHV-5 can be detected in the lungs of unaffected horses, but it has been associated with equine multinodular pulmonary fibrosis.[4,19] Similarly, the role of EHV-2 as a pathogen is controversial; some studies have demonstrated its association with upper respiratory tract disease, lymphadenopathy, immunosuppression, and keratoconjunctivitis.[5,13,22] Unfortunately, relatively few respiratory cases used in our study had pathology results available (either cytologic or histologic), making interpretation of the findings difficult. Interpretation of any molecular test result, including qPCR and/or NGS, should take into consideration the clinical and pathologic findings (if available) for accurate diagnosis. However, producing an assay with the ability to detect the full range of pathogen shedding, including small amounts that would be expected in convalescent cases, is desirable. At least for some pathogens that we tested, qPCR performed better for detection of very low levels of pathogen.

Most enteric cases had good correlation between microbiologic testing techniques, targeted NGS results, and pathologic findings. However, in 2 enteric cases, in addition to the *C. perfringens* toxins that were detected by both the new assay and qPCR, our targeted NGS detected some additional pathogens. These included *E. coli* hemolysin (Hly) virulence factor in intestinal scrapings from a horse with gastric ulcers, and *E. coli* cytotoxic necrotizing factor 1 (CNF-1) and intimin (eae) virulence factors in intestinal scrapings from a horse with necrohemorrhagic enterocolitis, which also had concurrent *C. perfringens* alpha toxin and *Cryptococcus* sp. detected by targeted NGS. Although *C. perfringens* was detected by qPCR (Ct 34.7), the reason the other pathogens were missed by routine methods is because the specific test needed was not performed or not requested on sample submission. Although the multiple pathogens identified by targeted NGS may represent mixed infection with multiple virulent organisms contributing to clinical disease, additional samples correlating pathologic findings with the presence of virulent pathogens are needed before clinical interpretations can be made.

As demonstrated with our targeted NGS results, an advantage of this method is its ability to not only detect conserved regions of these enteric pathogens but to identify *E. coli* and *C. perfringens* virulence factors or toxins to distinguish commensals from pathogenic bacteria. Other potential advantageous applications of NGS methods are to identify genotypic markers of drug resistance and virulence, as well as strain typing. Therefore, targeted NGS could distinguish between wild-type and vaccine strains as well as predict phenotypic antimicrobial resistance by targeting known genetic determinants of antimicrobial resistance.[25-27] Detection and characterization of the pathogen from the clinical sample could be done in a single assay.

Overall, our targeted NGS testing method performed favorably, and our pilot study has demonstrated the feasibility of using targeted NGS for equine infectious disease testing. However, redesign of the primer pools for targets with reduced sensitivity as well as further evaluation are recommended to improve the assay.

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Supplementary material

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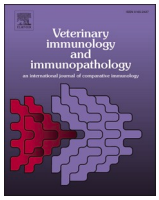

Research paper

Horses affected by EPM have increased sCD14 compared to healthy horses

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ABSTRACT

Equine protozoal myeloencephalitis (EPM) is a debilitating neurologic disease affecting horses across the Americas. Gaps in understanding the inflammatory immune response in EPM-affected horses create difficulties with diagnosis and treatment, subsequently negatively impacting the prognosis of affected horses. The purpose of the current study was to evaluate circulating levels of the inflammatory immune marker soluble CD14 (sCD14), in horses with EPM (n = 7) and determine if they differed from healthy neurologically normal horses (n = 6). Paired sera and cerebrospinal fluid (CSF) samples were analyzed for sCD14. Inclusion criteria for EPM horses consisted of the presence of neurologic signs consistent with EPM, *Sarcocystis neurona* surface antigens 2, 4/3 (SnSAG 2, 4/3) ELISA serum: CSF antibody ratio $\leq$ 100, and a postmortem diagnosis of EPM. Control horses were neurologically normal, healthy horses with SnSAG 2, 4/3 ELISA serum: CSF antibody ratios of $>$ 100. Serum anti-*Sarcocystis neurona* antibodies indicate that healthy control horses were exposed to *S. neurona* but resistant to developing clinical EPM. EPM cases had significantly greater concentrations of sCD14 in CSF samples compared to control horses and increased serum sCD14 concentrations. A positive correlation between sCD14 serum and CSF concentrations was observed in EPM-affected horses but not healthy horses. Soluble CD14 is an inflammatory marker, and the study results suggest it is elevated in EPM patients. When performed in conjunction with clinical evaluation and standard antibody testing, there may be potential for sCD14 to be utilized as a correlate for EPM.

Abbreviations: CNS, Central Nervous System; CRP, C-Reactive Protein; CSF, Cerebrospinal Fluid; CVSM, Cervical Vertebral Stenotic Myelopathy; EPM, Equine Protozoal Myeloencephalitis; HIV, Human Immunodeficiency Virus; SAA, Serum Amyloid A; sCD14, Soluble CD14; SnSAG, *Sarcocystis neurona* surface antigen.

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1. Introduction

Equine protozoal myeloencephalitis (EPM) is a common neurologic disease amongst horses in the Americas, predominantly caused by the protozoa *Sarcocystis neurona*, and affecting approximately 1% of the equine population in North America (Dubey et al., 1991; Dubey and Lindsay, 1998; NAHMS, 2001). Seroprevalence of *S. neurona* antibodies indicates exposure to *S. neurona* in 15–89 % of tested horses depending on geographical region (Reed et al., 2016). The majority of exposed horses presumably mount a protective immune response and remain neurologically normal. However, an aberrant, non-protective immune response is suspected in horses that develop clinical neurologic disease (Lewis et al., 2014; Spencer et al., 2004; Tornquist et al., 2001; Witonsky et al., 2008; Yang et al., 2006). Clinical neurologic disease results from *S. neurona* parasitizing the central nervous system (CNS), creating focal or multifocal inflammatory lesions within the CNS of affected horses. Since *S. neurona*-associated inflammatory lesions can occur within any region of the CNS, infection can cause variable clinical neurologic signs including muscle atrophy, proprioception loss, cranial nerve deficits, and others (Dubey et al., 2015). The variety of clinical neurologic signs associated with EPM contributes to the difficulty of diagnosing the disease. To further complicate diagnosis, the ratio of serum and CSF *S. neurona* antibodies remains the most specific antemortem diagnostic tool (Yeargan et al., 2015). However, clinicians are often limited to serologic testing due to the financial burden of CSF testing and the technicality of performing CSF taps outside of a clinical hospital setting.

On postmortem examination, EPM-associated CNS lesions are characterized by inflammatory changes, indicating a pathological role for inflammation. However, antemortem evaluation and characterization of the inflammatory response and its diagnostic utility require further investigation (Dubey et al., 1991; Furr et al., 2006; Mittelman et al., 2018; Reed et al., 2016; Rooney et al., 1970). Previous reports found that the acute phase inflammatory proteins, serum amyloid A (SAA), and C-reactive protein (CRP) serve little differential diagnostic value as serum and CSF concentrations did not significantly differ between EPM-affected horses and horses afflicted with other neurologic diseases (Mittelman et al., 2018). Conversely, phosphorylated neurofilament H (pNF-H), a biomarker associated with neuronal degeneration, has been reported to differ significantly between horses with EPM, horses with cervical vertebral stenotic myelopathy (CVSM), and neurologically normal horses (Intan-Shameha et al., 2017); this difference can be attributed to inflammation in the CNS (neuroinflammation) causing increased neuronal degeneration.

CD14 is a membrane bound co-receptor predominantly expressed on monocytes and macrophages and, to a lesser extent, neutrophils and B-lymphocytes (Shive et al., 2015; Ziegler-Heitbrock et al., 1994). CD14 can be cleaved from the cell membrane upon activation and released into circulation as sCD14, or the soluble form of CD14 can be secreted from intracellular sources (Shive et al., 2015; Ulevitch and Tobias, 1995). Elevated sCD14 CSF concentrations have been observed in numerous human diseases including human immunodeficiency virus (HIV) patients with neurocognitive impairment (Jespersen et al., 2016), bacterial meningitis (Cauwels et al., 1999), and multiple sclerosis (Harris et al., 2017). In the horse, serum sCD14 concentrations have been reported to be elevated in horses hospitalized due to inflammatory conditions (Perkins et al., 2019; Silva et al., 2013; Wagner et al., 2013; Ziegler et al., 2019), but to the best of the authors' knowledge, there are no reports regarding sCD14 changes in horses with neurologic diseases. The current pilot study was designed to determine if serum and CSF sCD14 concentrations differed between EPM-affected horses and healthy horses, and to assess the feasibility of this biomarker to aid in the diagnosis of EPM. It was hypothesized that sCD14 concentrations would be elevated in EPM-affected horses compared to healthy horses due to the inflammatory nature of the disease.

2. Materials and methods

2.1. Horses

Paired serum and CSF samples were selected from a collection of samples for an ongoing study and stored at −80 °C until analysis. For both the EPM group (n = 7) and control group (n = 6), the selected samples were from a mixed breed and mixed gender (4 female and 9 castrated male) population of horses with the average age of 13.4 years (± 4.2 years) for the EPM group and 16 years (±6.1 years) for the control group. For the EPM group, a confirmed EPM diagnosis was determined based on neurologic abnormalities consistent with EPM and assessment utilizing the Mayhew neurologic scale (Mayhew, 1989), serum: CSF *S. neurona* antibody ratio ≤100 using SnSAG 2, 4/3 ELISA testing (Equine Diagnostic Solutions, Lexington, KY)(Yeargan et al., 2015), and postmortem examination of the CNS revealing pathological lesions consistent with EPM (Dubey et al., 2015). Neurologic abnormalities observed in the EPM group included muscle atrophy, ataxia, and proprioception loss. For the treatment of horses in the EPM group (n = 7), 3 of 7 did not receive antiprotozoal treatment, 2 of 7 horses were undergoing treatment with an antiprotozoal at the time of presentation for the current study, and 2 of 7 received previous antiprotozoal treatment but were not being treated at the time of presentation for the current study. The control group consisted of neurologically normal horses with no current history of inflammatory disease or recent administration of anti-inflammatory medication. They had serum: CSF *S. neurona* antibody ratios >100 using SnSAG 2, 4/3 ELISA testing (Equine Diagnostic Solutions, Lexington, KY), and post-mortem examination of the CNS revealed no pathological changes. *Sarcocystis neurona* antibody ratio results indicated that horses in the control group had been exposed to *S. neurona*; however, had no previous history of clinical EPM suggesting disease resistance in this group. Neurologic exams were performed, blood was collected from each horse by jugular venipuncture with sera removed after centrifugation and stored at −80 °C for future analysis. CSF was collected from the atlanto-occipital space following euthanasia, and stored at −80 °C for future analysis. Following euthanasia, necropsies were performed. All horses included in this study were donated to the Virginia Maryland College of Veterinary Medicine (Blacksburg, VA) for this study. All sample collection was carried out in accordance with the Virginia Tech IACUC (protocol number 18-013), and client consent was obtained prior to patient enrollement.

2.2. Soluble CD14 analysis

The serum and CSF sCD14 concentrations were analyzed by a magnetic bead-based assay and analysis was conducted at the Animal Health Diagnostic Center at Cornell University (Ithaca, NY). The methodology for sCD14 analysis was previously described by Wagner et al., 2013. The sCD14 assay measures the respective analyte against a quantitative standard, and therefore both serum and CSF samples can be analyzed by the sCD14 assay. The monoclonal anti-equine CD14 antibody utilized for the sCD14 assay was previously validated (Wagner et al., 2013).

2.3. Statistical analysis

Normal distribution of the data was determined with a Shapiro-Wilk normality test. A parametric unpaired T-test with Welch's correction was conducted for analysis of sCD14 concentration comparison between the control and EPM groups for both serum and CSF samples. For serum and CSF correlation analysis, a Pearson correlation test was used to evaluate correlation significance. All analyses were performed using GraphPad Prism 8 v 4.1 (San Diego, CA, USA) and statistical significance cut off set at $P = < 0.05$.

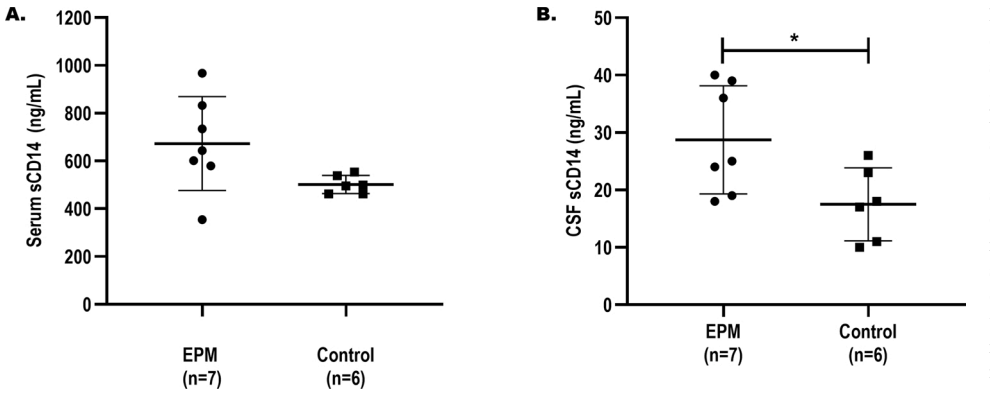

Fig. 1. Analysis of serum and CSF sCD14 concentrations.
sCD14 concentrations were compared in horses affected by EPM (n = 7) and healthy control horses (n = 6). A. There is a trend towards a significantly (P = .06) greater concentration (mean 673 ng/mL ±197 ng/mL SD) of serum sCD14 in EPM-affected horses compared to concentrations in control horses (mean 502 ng/mL ±38 ng/mL SD). B. The EPM group had significantly (P = .03) greater CSF sCD14 concentration (mean 29 ng/mL ± 9 SD) compared to concentrations in control horses (mean 18 ng/mL ±6 ng/mL SD). *P < .05. Error bars are represented as SD from the mean.

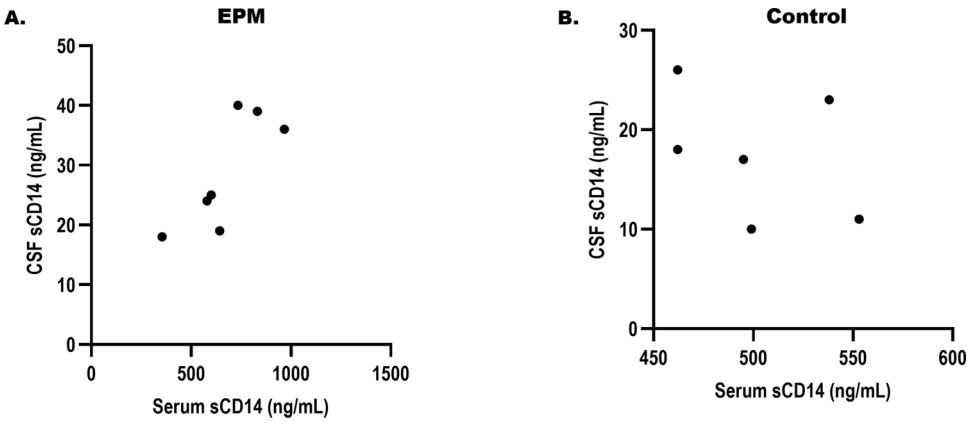

Fig. 2. The correlation of sCD14 concentration in paired serum and CSF samples.
A. There was a significant positive correlation (P = 0.03, r = 0.8) between paired and serum and CSF samples collected from EPM-affected horses (n = 7). B. There was no significant correlation (r= −0.39, P = 0.44) between paired serum and CSF samples collected from control horses (n = 6).

3. Results

In the EPM-affected horses (n = 7), serum sCD14 concentrations (mean concentration 673 ng/mL ±197 ng/mL SD) trended towards being significantly greater (P = 0.06) than control horse (n = 6) concentrations (mean concentration 502 ng/mL ±38 ng/mL SD) (Fig. 1A). The CSF sCD14 concentration (mean concentration 29 ng/mL ± 9 SD) was significantly greater (P = 0.03) in the EPM-affected horses compared to the control horses (mean concentration 18 ng/mL ±6 ng/mL SD) (Fig. 1B). There was a significant positive correlation between paired serum and CSF samples collected from the EPM-affected horses (r = 0.80, P = 0.03) (Fig. 2A). In contrast, paired serum and CSF samples collected from the control group did not correlate (r= -0.39, P = 0.44) (Fig. 2A). Collectively, these findings indicate a difference in circulating sCD14 concentrations between EPM-affected horses and healthy neurologically normal horses.

4. Discussion

EPM is a debilitating neurologic disease caused by an invasion of the CNS by *S. neurona* and presumably the resulting inflammatory response. The disease is notoriously difficult to definitively diagnose due to the lack of conclusive serological testing and non-specific clinical signs. In the current study, we evaluated concentrations of the inflammatory biomarker, sCD14, in both serum and CSF samples collected from EPM-affected horses and healthy neurologically normal horses. We found that EPM-affected horses had higher concentrations of sCD14 compared to healthy control horses in the serum and significantly higher in the CSF. The significant positive correlation between serum and CSF samples collected from EPM-affected horses indicates a relationship between peripheral and intrathecal sCD14 concentrations. In the current study, a correlative relationship was found to be specific to the EPM group and was not observed in the control horses, further suggesting a potential role for sCD14 in distinguishing EPM-affected horses from healthy neurologically normal horses. It remains to be determined whether the observed sCD14 relationship is a result of passive diffusion of sCD14 through the blood brain barrier or a result of increases in both peripheral and intrathecal sCD14 production. As previously mentioned, elevated sCD14 CSF concentrations have been observed in numerous human diseases, including human immunodeficiency virus (HIV), patients with neurocognitive impairment (Jespersen et al., 2016), bacterial meningitis (Cauwels et al., 1999), and multiple sclerosis (Harris et al., 2017). Additionally, in humans, it has been demonstrated that resident CNS macrophages (microglia) are a cellular source of sCD14 (Yin et al., 2009). Currently, it is unknown whether or not microglia are a cellular source of sCD14 in horses. In horses, previous studies have reported that sCD14 concentrations are elevated in plasma, serum, or colostrum samples collected from horses with inflammatory conditions, such as sepsis, recurrent airway obstruction, and gastrointestinal diseases when compared to healthy control horses (Silva et al., 2013; Wagner et al., 2013; Ziegler et al., 2019).The role of inflammation in EPM immunopathogenesis has previously been demonstrated in a study reporting upregulated gene expression of the proinflammatory cytokines, *IL-8*, *TNFA*, and *IFNG* in EPM-affected horses compared to control horses (Pusterla et al., 2006a), and it has been suggested that cytokine gene expression profiles differ amongst equine infectious and non-infectious neurologic diseases (Pusterla et al., 2006b). Additionally, a previous report indicated that the neuronal degeneration biomarker, pNF-H,

differed significantly between EPM-affected horses and neurologically normal horses (Intan-Shameha et al., 2017).

The current study is the first to suggest the role of sCD14 in an equine neurologic disease and its potential to aid in the characterization of the inflammatory response associated with inflammatory neurologic diseases such as EPM. Soluble CD14 is a myeloid derived innate immune cell inflammatory marker and is correlated with monocyte activation (Bas et al., 2004; Shive et al., 2015). The elevated sCD14 concentrations in EPM-affected horses suggest that pathogenic inflammation associated with EPM may at least be partially attributable to monocyte /macrophage activation and potentially activation of resident CNS macrophages, microglia. Previous studies have reported the infiltration of monocytes by *S. neurona* merozoites *in vitro* (Lindsay et al., 2006) and *S. neurona* schizonts and merozoites have been identified in macrophages in CNS inflammatory lesions (Dubey et al., 2015, 2001). Characterization of the peripheral inflammatory and neuroinflammatory response associated with EPM will provide knowledge to expand treatment options to include appropriate immunomodulators and improve the prognosis for horses with EPM.

Additionally, the results of the current study suggest a potential role of sCD14 as a supplementary diagnostic marker that maybe beneficially utilized in conjunction with standard serum and CSF *S. neurona* antibody tests to diagnosis EPM. The current study did not investigate the potential use of sCD14 as a differential diagnostic marker to differentiate EPM from other inflammatory and non-inflammatory neurologic diseases. Given the non-specific nature of sCD14 as an inflammatory marker, it is possible that other inflammatory neurologic diseases such as CVSM or West Nile virus, may also result in increased sCD14 serum and/or CSF concentrations; therefore, future studies are warranted. The results of the current study did not indicate a significant difference in serum concentration between EPM and control horses there was a trending difference. A supplementary and/or differential diagnostic marker, especially a serological marker, would have an immense clinical impact and drastically improve false diagnosis of EPM that often occurs as a consequence of only testing serum *S. neurona* antibody titers (Johnson et al., 2013; Yeargan et al., 2015). Although the current study was limited by sample size, our findings suggest a role of sCD14 in the diagnosis and pathogenesis of EPM.

In summary, the findings from the current study indicate a difference in circulating sCD14 concentrations between horses with EPM and healthy neurologically normal horses. Based on the results from the current study EPM-affected horses have greater CSF and serum sCD14 concentrations compared to healthy horses. Our study results indicate that there is potential for sCD14 to be used as a correlate for EPM diagnosis along with standard *S. neurona* serum and CSF antibody testing. Furthermore, since sCD14 is a general marker of systemic inflammation and monocyte activation, future studies investigating the monocyte/macrophage populations in EPM affected horses are warranted.

IACUC

This study was conducted in accordance with Virginia Tech's Institutional Animal Care and Use Committee (IACUC #15-070).

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Declaration of Competing Interest

The authors do not have any conflicts of interest.

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WILEY

Ultrasonographic and computed tomographic features of rice bodies in an Arabian horse with atlantal bursitis

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Abstract

A 19-year-old castrated Arabian male horse presented for evaluation of a firm mass at the dorsal cervical region. Ultrasonography and computed tomography revealed multiple well defined fusiform structures within the atlantal bursa. Multiple glossy smooth, white to yellowish, flattened fusiform structures were removed surgically. These structures were composed of dense fibrin with some leukocytes and red blood cells. The imaging and histopathological features of these structures were similar to chronic 'rice bodies' reported in humans with bursitis or tenosynovitis. This is the first veterinary report describing the imaging features of 'rice bodies' in a horse with atlantal bursitis.

KEYWORDS

bursa, cervical, equine, tenosynovitis

1 | SIGNALMENT, HISTORY, AND CLINICAL FINDINGS

A 19-year-old castrated Arabian male horse was presented to the Purdue University Veterinary Teaching Hospital for evaluation of a firm, soft tissue mass at the dorsal aspect of the cranial cervical region. According to the owner, the mass had gradually grown in size over the past 3 years and has doubled in size over the past 2 months. Upon physical examination, a large and firm soft tissue mass was present extending over both sides of the poll immediately behind the occiput with greater swelling on the right side of the poll. No pain was elicited on palpation and no heat was noted in the area. The horse was reluctant to flex the poll and to be tied.

2 | IMAGING, DIAGNOSIS, AND OUTCOME

Standing laterolateral radiographs of the cranial cervical spine were taken (Canon IP CXDI-80C, Tokyo, Japan; Vet Rocket LLC reader, California, USA; 80 kVp and 15 mAs). There was a focal soft tissue swelling dorsal to the atlas (C1), with an oval area (7.4 x 19 cm) of circumscribed stippled mineralization within it. The atlantal crest of the occiput showed a focal area of a palisading periosteal reaction (Figure 1). Radiological findings were suggestive of chronic, severe atlantal bursitis with dystrophic mineralization and occipital periostitis, or less likely a large chronic mineralized hematoma.

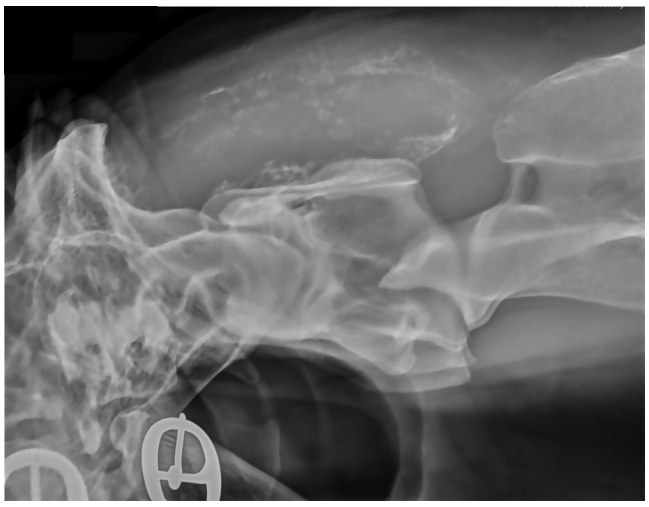

FIGURE 1 Standing laterolateral radiograph from the occipital bone extending to the mid body of axis. There is a well circumscribed area of mineralization at the atlantal bursa region with a focal area of palisading periosteal reaction at the occiput

Ultrasonography (MyLab 70 XVG, Biosound Esaote, Italy; LA523 linear transducer with 4–13 MHz bandwidth) was performed for evaluation and sampling of the content within the atlantal bursa. The bursa contained a moderate volume of anechoic fluid and multiple, stacked,

*Primary author

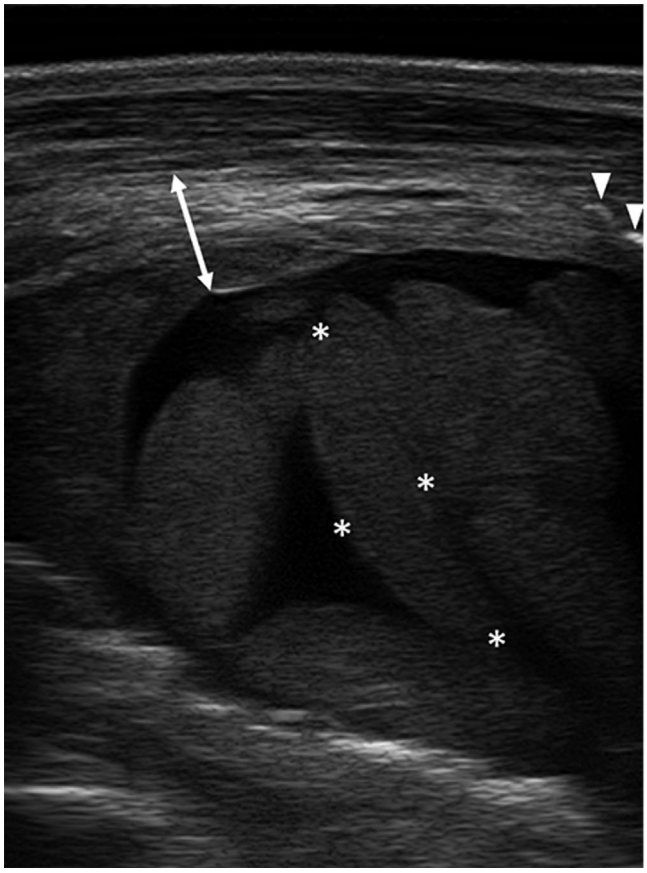

FIGURE 2 Ultrasound image of the atlantal bursa. Thickened bursal wall (double-sided arrow) with few short curvilinear hyperechoic mineral specks (white arrowheads). Rice bodies are present within the fluid-filled bursa (one outlined with asterisks)

fusiform structures that were mildly hyperechoic to the cervical musculature. The wall of the bursa was thickened up to 1.7 cm. The outer wall of the bursa was hyperechoic with few short curvilinear hyperechoic mineral specks within. The inner wall of the bursa was mildly hypo- to isoechoic to the cervical musculature (Figure 2). The serology test for Brucellosis was negative. Cytology of synovial fluid from the atlantal bursa showed a chronic inflammatory process with negative bacterial culture.

For presurgical planning, noncontrast helical computed tomography (CT) (GE LightSpeed VCT 64-Slice, Milwaukee, Wisconsin; 140 kV, 98 mAs, 2.5 mm slice thickness in detail algorithm with reconstruction interval of 1.25 mm, tube rotation time 1 s, and pitch of 1.0) of the cranial cervical region (from caudal occiput to mid axis) was performed under general anesthesia with the horse in right lateral recumbency. Dorsal and sagittal multiplanar reconstruction with soft tissue and bone windows was performed to further assess extent of involvement. The caudal and cranial aspects of the atlantal bursa contained large numbers of stacked fusiform, free-floating soft tissue attenuating structures (Hounsfield unit (HU) 30–50) of variable sizes interposed with small amounts of fluid attenuating material (HU 10–20). The wall of the bursa was irregularly mineralized (Figure 3).

Numerous glossy smooth, white to yellowish flattened fusiform structures (up to 4 x 2 cm each) with yellowish fibrinous material were removed via atlantal bursotomy (Figure 4A). A small section of the wall of the bursa was submitted for culture and sensitivity. No aerobic bacterial growth was yielded. The fusiform structures together with the yellowish fibrinous material within the bursa were submitted for histopathological analysis. On histopathology, the fusiform structures were composed of poorly cellular necrotic and eosinophilic tissue admixed with fibrin, red blood cells, and rare fibroblasts (Figure 4B–D). The presence and formation of these fusiform structures were likely due to chronic inflammatory process of unknown cause. The ultrasonographic, CT, and gross appearance of the numerous fusiform structures within the atlantal bursa as well as the histopathological findings were similar to intra-articular rice bodies reported in human literature and hence the diagnosis of rice bodies atlantal bursitis was made.[1–3]

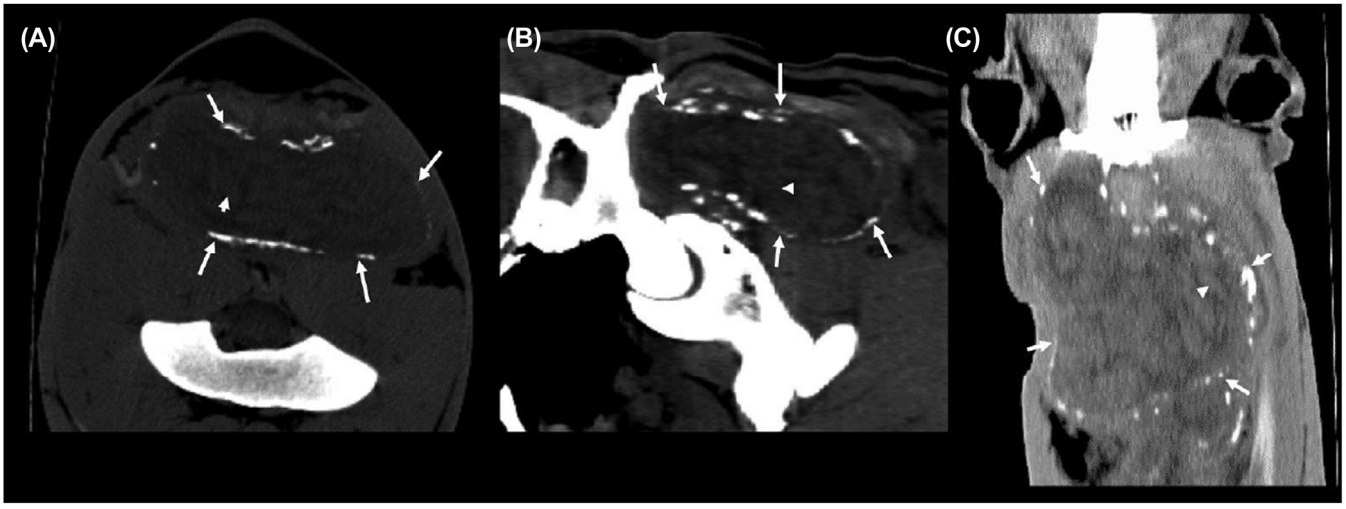

FIGURE 3 Multiplanar reconstructed (MPR) CT images of the atlantal bursa obtained in a soft tissue window. (A) Transverse image, (B) sagittal MPR image, and (C) Dorsal MPR image. Mineralized bursal wall is visible in all three images (white arrows). Multiple rice bodies (HU 30–50) were present within the bursal lumen and surrounded by hypoattenuating fluid (HU 10–20) (white arrowheads). Irregular periosteal new bone is present on the occiput on dorsal MPR image. The windowing has been adjusted to provide optimal visualization of the rice bodies

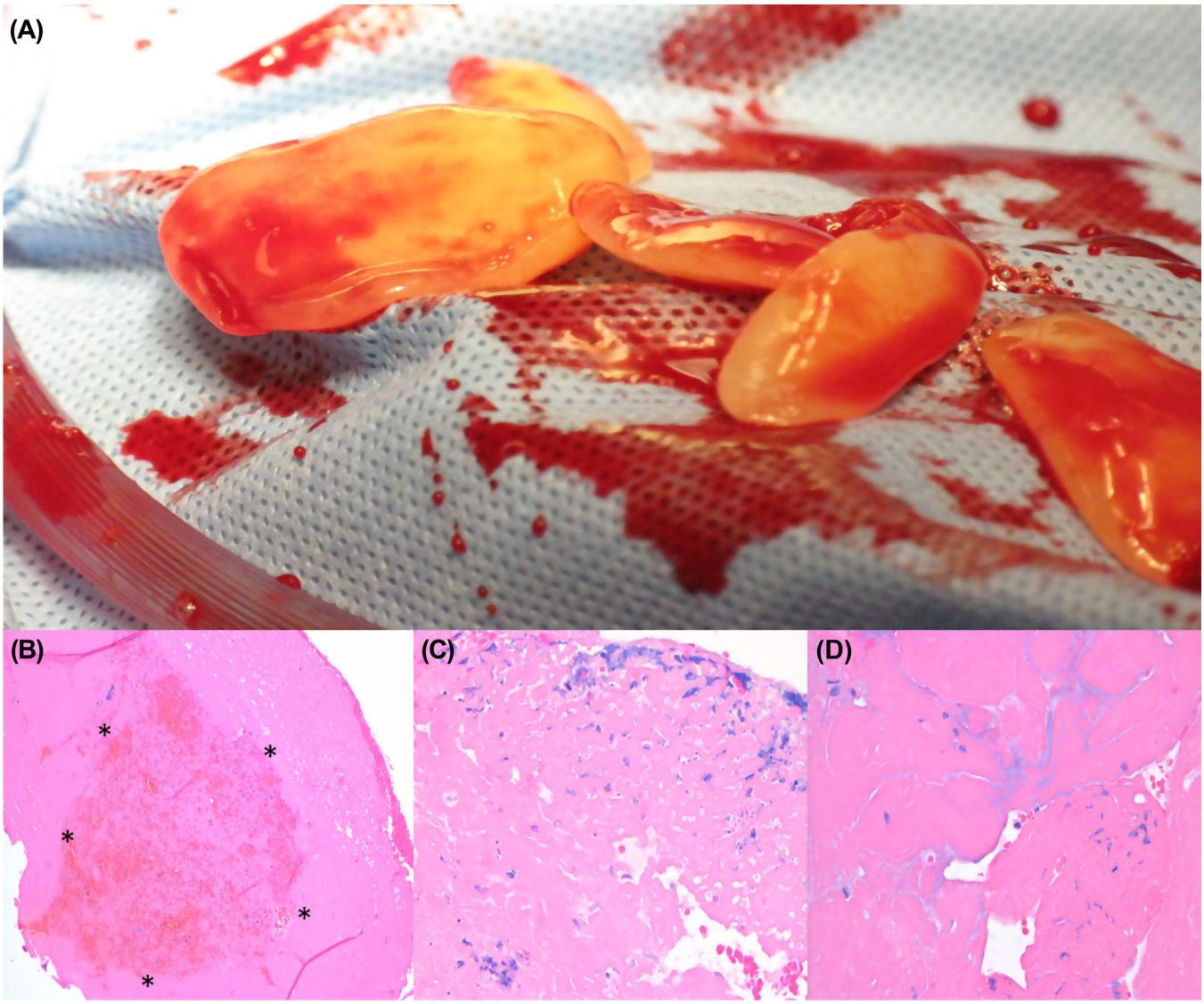

FIGURE 4 (A) Gross image of the rice bodies removed from atlantal bursotomy. (B) Histopathologic section of a rice body stained with H&E stain, containing fibrin and erythrocytes (asterisks outlining the erythrocytes). (C) and (D): The rice bodies appear flattened. The brightly eosinophilic and amorphous material is fibrin. The blue staining is mostly remnants of nuclear material from degenerated cells as well the nucleus of some fibroblasts (black arrowheads)

The horse has been used for pleasure and therapeutic riding for the past 3 years since recovering from surgery, with no complications or regrowth of the mass.

3 | DISCUSSION

This paper is the first in veterinary literature that describes the radiographic, ultrasonographic, and CT findings for histologically confirmed "rice bodies" in a horse with atlantal bursitis. One case of rice bodies has been reported in the stifle of a draft stallion without any imaging description.[4] Intra-articular rice bodies have been reported extensively in human literature.[1–3,5,6] Morphologically, human rice bodies are free-floating oblong structures with a glossy surface within the joint capsule and bursae.[1,5]

The fusiform structures identified within the atlantal bursa were white to yellowish in color, smooth, glossy, and free-floating within the fibrinous fluid, grossly similar to rice bodies described in human literature.[1,5–7] These were also histopathologically similar to human rice bodies, composing of tightly packed fibrin and collagen with a mixture of intermittent fibroblasts and red blood cells.[2]

Rice bodies bursitis has been described as multiple stacking oblong-shaped filling defects by positive bursography in the human.[6] Although positive bursography was not performed in our case, we expect to yield similar fusiform shaped filling defects if we have done so. The oval-shaped stippled mineralization seen on our radiographs corresponds with dystrophic mineralization of the thickened wall of the atlantal bursa seen on CT. Mineralization of the synovium or joint capsule was not observed in human.[5–7] The chronicity of the atlantal bursitis (over the duration of 3 years) may have led to thickening and synovial proliferation of the bursal wall, with subsequent fibrosis and dystrophic mineralization.

Ultrasononography was found to be useful in depicting the appearance and shape of the rice bodies in this case with minimal invasion and also facilitated the sampling of the synovial fluid. While the ultrasonographic appearance of the rice bodies within the atlantal bursa were similar in shape (fusiform) and echogenicity (isoechoic to the muscle) to the intra-articular rice bodies described in human literature,[1,3] the rice bodies in our case were relatively much larger in size (up to 4 cm in diameter vs. 1 cm diameter in humans). This may be attributable to the relatively larger size and volume of the atlantal bursa of a horse compared to those bursae found in the human radius and ulna. The size of these rice bodies measured on ultrasound also corresponded well with the CT and direct measurements of the gross samples acquired from the bursotomy.

The previous CT case reports on humans with chronic bicipitoradial and subacromial bursitis failed to detect these individual rice bodies.[7] Instead, CT of these rice bodies and bursae only showed distended bursa with homogeneous fluid to soft tissue attenuation within, with a mild rim of enhancement of the wall of the bursa.[7] We were unable to assess for rim enhancement of the wall of the atlantal bursa as no contrast agent was administered. The well-defined oblong-shaped rice bodies in human cases were only visible on magnetic resonance imaging, with the rice bodies being almost isointense with the musculature on T1W and T2W sequences.[3,7] On the contrary, we were able to visualize the rice bodies within the atlantal bursa, likely due to the relative larger size of rice bodies in the horse and improved CT technology to provide better image quality compared to the CT technology two decades ago. In our case, presurgical CT aided the surgeons to estimate the true extent and dimensions of the affected atlantal bursa as well as for surgical planning.

The pathophysiology of rice bodies formation has been theorized to include synovial chondromatosis, chronic inflammation secondary to trauma of the joint space, rheumatoid arthritis, and tuberculosis infection.[1,5,6,8,9] Regardless of the underlying cause of human rice bodies, they have a similar fusiform to discoid appearance. For rheumatoid arthritis, it has been hypothesized that the cells that made up the rice bodies were initially cells from synovial origin that had sloughed from the synovium from microinfarction secondary to bursal hypoxia. These cells subsequently developed into free moving bodies within the joint.[10,11] Continuous immune mediated response and synovitis perpetuates arthritis and further tissue hypoxia in the joint, making this a self-perpetuating cycle of rice bodies formation.[11] Presence of larger rice bodies has been reported in humans with tuberculosis arthritis compared to those with rheumatoid arthritis.[12] We attribute the larger sized rice bodies to the greater size of the equine atlantal bursa.

We speculate that the most likely cause of the rice bodies formation in this horse was due to chronic inflammation or the fragmentation of part of the synovial surface that served a nidus for formation of these structures. This would be consistent with the prolonged duration and gradual enlargement of the atlantal bursal mass over time in our case. Although mycobacterial tenosynovitis and bursitis with rice bodies have been reported in humans,[1] it was unlikely in our case as the bursal samples of the horse yielded negative result for mycobacterial culture. Removal of the rice bodies with debridement of the thickened bursal wall via surgical bursotomy appears to have good outcome as indicated in the present case, with no evidence of recurrence.

We conclude that the rice bodies in this horse with atlantal bursitis had similar ultrasonographic and CT characteristics to those reported in humans. The findings of multiple fusiform rice bodies within a bursa on ultrasonography and CT should prompt the clinician to include chronic bursitis as a differential diagnosis in horses.

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Category 1

(a) Conception and Design: Hohu KK, Lim CK

(b) Acquisition of Data: Lim CK, Adams SB, Ramos-Vara JA

(c) Analysis and Interpretation of Data: Lim CK

Category 2

(a) Drafting the Article: Hohu KK, Lim CK

(b) Revising Article for Intellectual Content: Lim CK, Adams SB, Heng HG, Ramos-Vara JA

Category 3

(a) Final Approval of the Completed Article: Hohu KK, Lim CK, Adams SB, Heng HG, Ramos-Vara JA

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CASE REPORT

WILEY

Evaluation of equine corneal disease using ultrasound biomicroscopy

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Abstract

Objective: To describe the clinical use of ultrasound biomicroscopy (UBM) to evaluate equine corneal disease.

Methods: Images were obtained using a 50-MHz probe ultrasound biomicroscopy system (Quantel Aviso) and Clear Scan® probe cover. Six horses with corneal disease were evaluated via UBM for lesion size, lesion depth, and continuity of Descemet's membrane. Horses were sedated and received auriculopalpebral nerve blocks and application of topical anesthetic prior to UBM.

Results: Ultrasound biomicroscopy was easily performed in all cases. UBM evaluation of three cases of corneo-limbal squamous cell carcinoma yielded information regarding lesion depth for planning of keratectomies using fixed-depth keratomes and subsequent β-radiation therapy. Corneal depth and continuity of Descemet's membrane were determined in two horses with stromal abscesses and allowed for planning of therapeutic options. In one horse with a corneal foreign body, UBM contributed to accurate assessment of the foreign body's stromal depth, which could not be assessed during ophthalmic examination due to extensive corneal cellular infiltrate. The information regarding corneal depth allowed for more accurate pre-surgical planning in patients with opaque corneal lesions.

Conclusions: Ultrasound biomicroscopy was easily performed and provided useful information regarding lesion depth and continuity of Descemet's membrane for patients with corneo-limbal squamous cell carcinoma, stromal abscesses, and a corneal foreign body, allowing for increased precision in pre-surgical planning and development of therapeutic protocols.

KEYWORDS

cornea, corneal foreign body, equine, squamous cell carcinoma, stromal abscess, ultrasound biomicroscopy

Presented in abstract form at the 47th Annual Conference of the American College of Veterinary Ophthalmologists, Monterey, CA, October 2016.

1 | INTRODUCTION

Corneal diseases such as neoplasia and stromal abscesses occur frequently in horses.[1] Assessing lesion depth can be critical for determining treatment options and prognosis. However, corneal opacities may obscure visualization during slit lamp biomicroscopy.

Ultrasonography is a commonly performed imaging technique for evaluation of the equine eye.[2] However, the 7.5–10 MHz probes do not provide adequate resolution for evaluation of the cornea.[2] Ultrasound biomicroscopy (UBM) probes have frequencies between 50 and 60 MHz and resolutions of 20–80 μm.[3–5] While tissue penetration is only 5 to 10 mm, this depth and resolution is ideal for imaging the cornea.[3–5] Using UBM, one can obtain images with microscopic resolution, providing a valuable imaging modality for patients with opaque corneal lesions that interfere with complete examination.[6,7] Physician ophthalmologists use UBM for detection of intracorneal, intrascleral, subconjunctival, and intraocular foreign bodies after ocular trauma, and evaluation of keratoconus, dystrophies, scarring, and masses.[5,7,8] The information gained from UBM helps surgeons determine whether a penetrating keratoplasty is indicated versus a non-penetrating surgical approach.[9]

In dogs, UBM has been used to assess corneal disease, the iridocorneal angle, anterior segment neoplasia, and uveal cysts.[3,4,10] In the horse, a 50 MHz transducer was used to determine central corneal thickness,[11] and an eye with a limbal hemangiosarcoma was imaged with a 20 MHz probe to determine the depth of corneal involvement.[4]

The purpose of this case series was to describe the clinical use of UBM in evaluation of equine corneal disease.

2 | MATERIALS AND METHODS

Medical records were reviewed to identify horses in which UBM was used to evaluate corneal lesions at the Purdue University Veterinary Teaching Hospital from 2012 to 2016. All horses received an ophthalmic examination by a board-certified veterinary ophthalmologist (WT), including slit lamp biomicroscopy (Kowa SL-14, Kowa), applanation tonometry (Tonopen®, Reichert Technologies), and fluorescein staining. When further information regarding the extent and depth of lesions was needed, UBM was performed. All clients provided informed consent.

Horses were sedated with either xylazine (0.3–0.6 m/kg IV) (TranquiVed, VedCo) or detomidine (0.01 mg/kg IV) (Dormosedan, Zoetis), often-in combination with butorphanol (0.01 mg/kg IV) (Torbugesic, Zoetis). All patients received auriculopalpebral nerve blocks with 1–2 ml of 2% lidocaine (Vet One®) and application of topical anesthetic, 0.5% tetracaine (Bausch & Lomb) prior to UBM. Imaging was performed using the 50-MHz mechanical linear scan transducer of a commercially available ultrasound biomicroscopy system (Aviso, Quantel Medical). A single-use disposable ClearScan® probe cover (ESI Inc.) was filled with distilled water and placed over the probe.[12] The eyelids were manually parted, and the probe was placed on the cornea, with a drop of saline solution as the coupling agent, over the lesion of interest to evaluate lesion size, depth, and continuity of Descemet's membrane.[12] Scans were performed in sagittal and transverse planes.

Still and video ultrasonographic images were recorded for all horses and evaluated by a board-certified ophthalmologist (WT). When indicated, structures were measured with the ultrasound biomicroscopy system's internal calipers.

3 | RESULTS

From 2012 to 2016, there were six horses for which UBM was used to evaluate corneal lesions: three horses with corneolimbal squamous cell carcinoma, two horses with stromal abscesses, and one horse with a corneal foreign body. In all cases, images were obtained with standing sedation and the technique was well tolerated. Images were easily obtained with typical imaging times of 5 min. No corneal damage or other complications were noted.

3.1 | Squamous cell carcinoma (Cases 1–3)

3.1.1 | Case 1

A 5-year-old Belgian gelding was referred for evaluation of a temporal corneal mass of the left eye (OS) confirmed as a squamous cell carcinoma (SCC) via biopsy by the referring veterinarian one week before presentation. Examination revealed a pink, irregular, raised, plaque-like corneal mass at the temporal limbus measuring 10 x 15 mm. The corneal depth could not be determined with biomicroscopy, but was suspected to have extensive stromal involvement due to the size of the lesion. Using UBM, the SCC was found to only involve the superficial stroma (Figure 1). Therefore, instead of enucleation, a superficial keratectomy and adjacent conjunctivectomy were performed under general anesthesia. Strontium-90 β-radiation was used at three corneal sites using 100 Gy per site. Histopathology confirmed SCC, but surgical margins at the keratectomy site could not be evaluated. Per the referring veterinarian, the surgical site healed well, but the patient was lost to follow-up after one month.

3.1.2 | Case 2

A 20-year-old Pony of the Americas gelding was referred for a rapidly growing pink mass at the temporal aspect of the right cornea. On examination, the lesion was pink, 5 x 2.5 mm, and plaque-like extending 3 mm beyond the

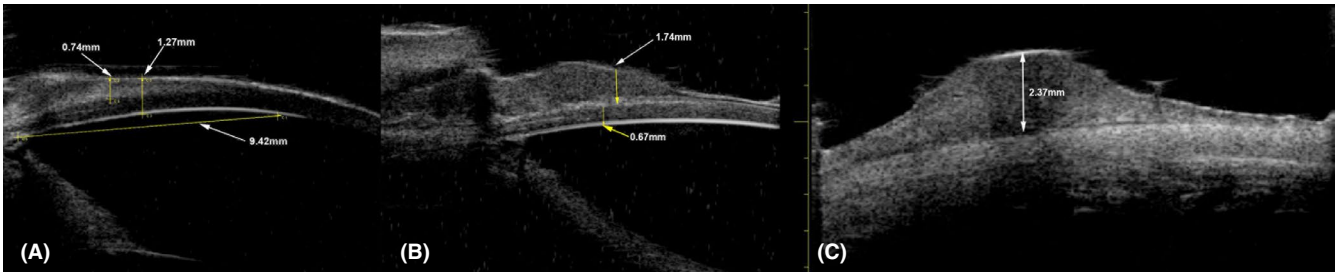

FIGURE 1 (A) Case 1—A 5-year-old Belgian with a limbal mass (squamous cell carcinoma—SCC) seen as a hyperechoic area (arrow, thickness of 0.74 mm) infiltrating into the superficial to mid stroma (corneal thickness of 1.27 mm) and extending 9.42 mm into the temporal cornea from the limbus. (B) Case 2—A 20-year-old Pony of the America's with a limbal SCC seen as a hyperechoic area (white arrow) extending into the corneal stroma with 0.67 mm of normal corneal stroma remaining. (C) Case 3—An 18-year-old crossbred mare with a raised mass (SCC) seen as a hypoechoic area on ultrasound (arrow, thickness of 2.37 mm) extending minimally into the superficial corneal stroma at the right side of the image

limbus onto the bulbar conjunctiva. Again, corneal depth could not be determined on routine examination. Pre-surgical UBM demonstrated that the mass extended to 20% stromal depth (Figure 1). Under general anesthesia, a superficial keratectomy at 25%–30% stromal depth was performed and the conjunctiva was excised 3 mm past the limbus down to the sclera. Following surgery, strontium-90 β-radiation was performed at three sites in the keratectomy bed for 100 Gy per site. Histopathology confirmed a well-differentiated squamous cell carcinoma with clean margins. In the 7 years since surgery, no regrowth has been noted.

3.1.3 | Case 3

An 18-year-old crossbred mare was presented for evaluation of a suspected corneal SCC. An unpigmented, ulcerated 5 mm plaque-like lesion on the temporal conjunctiva extending 2 mm into the temporal cornea was noted on examination OS. An excisional conjunctival biopsy performed with the Purdue Ophthalmology Service confirmed SCC, but neoplastic cells extended to the surgical margins. Eight days later, UBM was performed to evaluate the depth of the corneal lesion for presurgical planning (Figure 1). The lesion was very superficial, involving less than 10% of the corneal stroma. A 5 x 5 mm superficial keratectomy and conjunctivectomy were performed under standing sedation followed by two sites of strontium-90 β-radiation at 100 Gy per site. Margins could not be evaluated on the surgical sample submitted for histological examination. In the 6 years since surgery, no regrowth has been noted.

3.2 | Stromal abscess (Cases 4–5)

3.2.1 | Case 4

A 3-year-old Thoroughbred mare was referred for a one-week history of a stromal abscess OS. A 16 x 16 mm area of yellow stromal infiltrate was present in the axial cornea. Ultrasound biomicroscopy demonstrated a hyperechoic infiltrate extending into the deep stroma with Descemet's membrane intact (Figure 2). Medical therapy was elected by the owner due to the size of the lesion and surgical costs. Topical medical treatment included ofloxacin 0.3% ophthalmic solution, silver sulfadiazine 1% cream, atropine 1% ophthalmic solution, voriconazole 1% solution, and autologous serum. Oral medications included flunixin meglumine at 1.1 mg/kg q12 h and fluconazole at 14 mg/kg q24 h. Intrastromal voriconazole injections were performed twice along with three subconjunctival injections of 1 mg amphotericin B. At discharge 34 days later, the patient was comfortable with the abscess vascularized and stromal infiltrate regressing.

3.2.2 | Case 5

A 12-year-old Quarter Horse gelding presented for acute ocular pain and discharge OS. On examination, there was a dorsotemporal 6 x 6 mm area of yellow, deep stromal infiltrate with hypopyon extending from the abscess site into the anterior chamber (Figure 3). On UBM, the hyperechoic stromal infiltrate extended through a rupture in Descemet's membrane resulting in hypopyon. Based on these findings, a penetrating lamellar keratoplasty was performed with BioSISt^{TM} (Vetrix, Inc.) and a conjunctival graft placed. The gelding was discharged after 17 days and at the final recheck, 51 days after the initial examination, the patient was visual and comfortable.

3.3 | Corneal foreign body

3.3.1 | Case 6

A 2-year-old Quarter Horse mare was evaluated for a corneal ulcer OS after the referring veterinarian removed a corneal

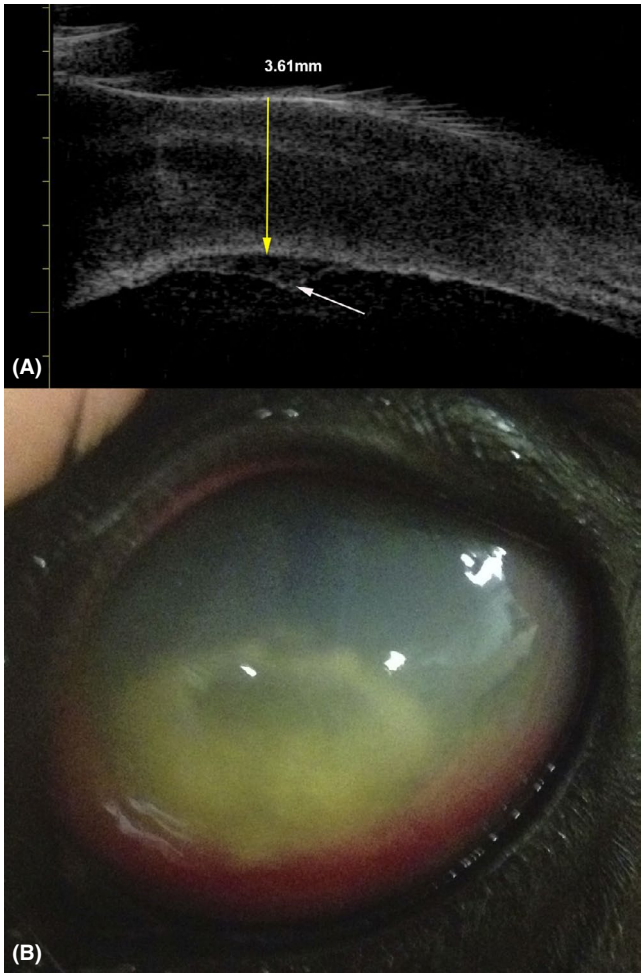

FIGURE 2 Case 4—(A) A 3-year-old Thoroughbred mare with the entire axial cornea in this image distended by hyperechoic material with a corneal thickness of 3.61 mm. An intact Descemet's membrane is visible at the tip of the yellow arrow. Fibrin can be seen as a wispy hyperechoic line extending from Descemet's membrane (white arrow). (B) A photograph of the extensive stromal abscess and peripheral corneal vascularization. The dense cellular infiltrates precluded visualization of Descemet's membrane

plant-based foreign body. A dorsotemporal corneal laceration with plant material embedded in the stroma was observed. The lesion had extensive corneal edema and cellular infiltrate, precluding assessment of the foreign body's depth.

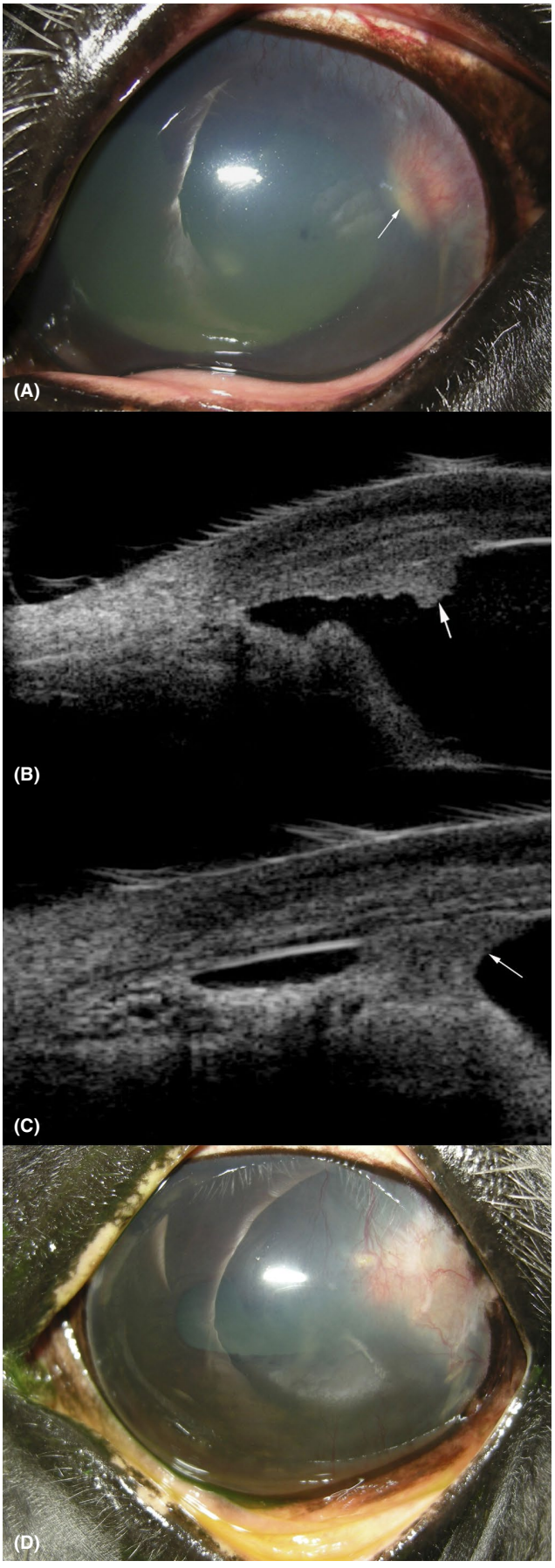

FIGURE 3 Case 5—(A) Photograph of a 12-year-old Quarter horse gelding with a dense stromal abscess (arrow) with overlying corneal vascularization. (B) Hyperechoic area of deep stromal infiltrates with loss of integrity of Descemet's membrane. Note the area of abscess extension into the anterior chamber (arrow). (C) Stromal abscess rupture through Descemet's membrane (arrow), resulting in hypopyon. (D) Photograph taken 37 days post-operatively. The cellular infiltrate has regressed. Corneal vascularization remains at the surgical site

Using UBM, hyperechoic retained foreign material was detected within the cornea extending from 600 to 1100 μm depth (~50% depth), with no evidence of Descemet's membrane rupture (Figure 4). Based on examination and UBM findings, a lamellar keratectomy at 50%–60% depth was performed, followed by a conjunctival pedicle graft. Focal keratomalacia with a plant foreign body was confirmed histologically. Aerobic bacterial culture revealed few *Enterococcus sp*. sensitive to topical ofloxacin which was continued post-operatively. The mare was discharged three days after the surgery and 14 days after the initial examination the patient was visual and comfortable with the graft well incorporated.

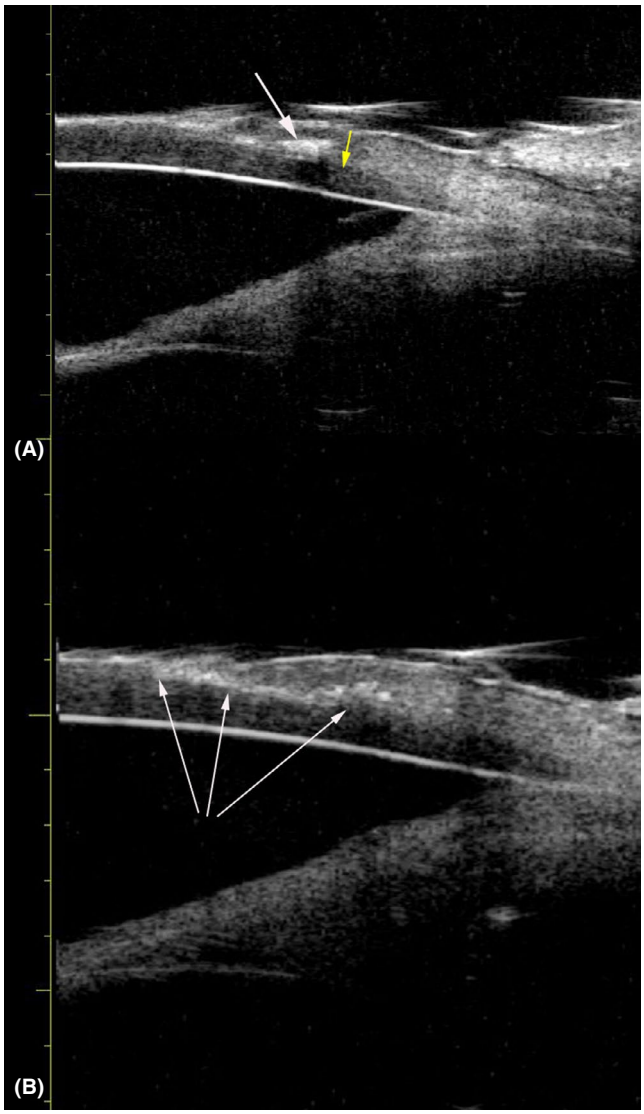

FIGURE 4 Case 6—A 2-year-old Quarter horse mare with a corneal foreign body. (A) A linear hyperechoic band demonstrates the presence of the plant-based foreign body (white arrow) which extends to 50% stromal depth (978 μm—yellow arrow). (B) The path of the corneal foreign body into the corneal stroma can be seen as the linear hyperechoic band (3 white arrows)

4 | DISCUSSION

In the present study, horses with corneal disease ranging from neoplastic to stromal abscess to foreign body were successfully evaluated using UBM. The examinations were well tolerated and performed with only standing sedation. Images were easily obtained with no complications.

Corneal neoplasia, such as the squamous cell carcinoma in Cases 1–3, is often treated via surgical resection and possibly additional therapy with cryosurgery or radiation. Often, the masses may extend deeper into the sclera or cornea, but extension can be difficult to assess with slit lamp biomicroscopy. Extensive lesions may warrant enucleation. Use of UBM allowed for evaluation of the degree of extension prior to surgical excision. Therefore, a more detailed surgical plan and prognosis could be discussed with the owners.

Corneal stromal abscesses are often treated with surgical removal via keratectomy. Corneal edema, neovascularization, and stromal infiltrates or plaques may decrease visualization of the deeper aspects of the cornea and the anterior chamber. Ultrasound biomicroscopy was successfully used in the cases reported here to determine the extent and depth of these opaque corneal lesions for surgical planning. The stromal infiltrates did not impair the ability to attain diagnostic images with UBM. Ultrasound biomicroscopy was also used to image the anterior chamber for signs of uveitis, such as fibrin and hypopyon. Use of UBM also allowed identification of a corneal foreign body and confirmed a break in Descemet's membrane.

The other advanced imaging modalities used to image the equine cornea are in vivo confocal microscopy[13–15] and spectral domain optical coherence tomography (SD-OCT).[16–18] Similar to confocal microscopy, UBM requires contact with the corneal surface which can be of concern when imaging a fragile cornea, whereas SD-OCT does not. Both confocal microscopy and SD-OCT can be time-consuming and movement of the horse can be challenging. UBM images are obtained much faster, and movement is less problematic. A UBM is highly portable, allowing stall side examination. A UBM also does not require modification before use in the horse. Unlike SD-OCT, UBM can image through dense corneal opacities.[16] However, UBM does not provide nearly the same level of resolution, particularly when compared to confocal microscopy.[13] Therefore, each imaging modality has different benefits and limitations. The one selected depends on the availability and the needs for each individual case. For identification of fungal hyphae, confocal microscopy would be superior.[14] For the highest resolutions, confocal microscopy and SD-OCT would be preferred. For rapid determination of lesion depth and assessment of the continuity of Descemet's membrane, UBM worked well in the cases reported here.

In conclusion, UBM provided useful information regarding lesion depth and continuity of Descemet's membrane for equine patients with corneo-limbal squamous cell carcinoma, stromal abscesses, and a corneal foreign body. This information allowed for development of therapeutic protocols and pre-surgical planning of keratectomies. The information garnered from the examination was also used to determine whether further tectonic surgical methods, such as a conjunctival graft, would be necessary as one could visualize preoperatively if the lesion extended into the posterior 50% of the stroma.

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CONFLICT OF INTERESTS

There is no conflict of interest to be disclosed.

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GENERAL ARTICLE

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Commercial amniotic membrane extract for treatment of corneal ulcers in adult horses

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Abstract

Background: Amniotic membrane extract enhances the rate of epithelialisation after corneal ulceration in several species but has not been studied in the equine cornea.

Objectives: To evaluate the effect of amniotic membrane extract on re-epithelialisation of equine corneal ulcers compared with ulcers treated with antibiotic, antifungal and mydriatic medical therapy alone, and to evaluate equine corneal healing after experimentally induced superficial ulceration.

Study design: Masked, randomised, controlled experimental trial.

Methods: Superficial, 8 mm corneal ulcers were created bilaterally in each horse. One eye was treated with amniotic membrane extract and the opposite was control. Both eyes were treated with medical therapy. Treatment eyes received amniotic membrane extract, and control eyes received the amniotic membrane extract vehicle. Ulcers were stained with fluorescein and photographed in 12-hour increments until completely healed. Ulcer surface area was determined by analysing photographs with ImageJ. A mixed linear model was used to compare ulcer surface area and hours until healing between treatment groups. A regression model was also used to calculate corneal re-epithelialisation rate over time.

Results: Regardless of therapy, healing occurred in two phases: an initial rapid phase of 0.88 mm^2/hr (95% CI: 0.81-0.94 mm^2/hr) for approximately 48-54 hours followed by a second, slow phase of 0.07 mm^2/hr (95% CI: 0.04-0.09 mm^2/hr). Most eyes healed within 135.5 ± 48.5 hours. Treatment (amniotic membrane extract vs. control) was not significantly associated with size of ulcers over time ($P = .984$). Discomfort was minimal to absent in all horses.

Main limitations: Results achieved experimental studies may differ from outcomes in the clinical setting.

Conclusions: There was no significant difference in healing rate with addition of amniotic membrane extract to medical therapy for equine superficial corneal ulcers. A biphasic corneal healing process was observed, with an initial rapid phase followed by a slow phase. Further study will be needed to determine whether amniotic membrane extract will be helpful for infected or malacic equine corneal ulcers.

KEYWORDS

horse, amniotic membrane extract, diamond burr, ulcer

The abstract is available in German in the Supporting Information section of the online version of this article.

1 | INTRODUCTION

Corneal ulceration occurs commonly in horses. Accelerating corneal healing decreases the risk of complications, diminishes stress and discomfort,[1] and potentially decreases treatment cost. A well-established method to assist corneal healing is using a tectonic graft composed of amniotic membrane.[2] Amniotic membrane promotes corneal epithelialisation and organised restoration of corneal stroma, inhibits corneal fibrosis and reduces corneal inflammation and neovascularisation in a rodent model.[1] Decreasing inflammation is particularly beneficial as uninhibited inflammatory mediators threaten the survival of stem cells, exacerbate neovascularisation and induce keratocyte apoptosis and stromal melting.[3] The beneficial effects are due to biomolecules within AM including fibronectin, hepatocyte growth factor, epidermal growth factor, basic fibroblast growth factor and collagen types I, III, IV and V.[4-6] The main limitation is the need for surgical application.

Commercial amniotic membrane extract allows for topical application and all beneficial effects of an amniotic membrane graft except tectonic support.[4,7-9] In the limited studies to date, human, rodent and rabbit corneal epithelial cell defects treated with amniotic membrane extract have healed significantly faster and with less inflammation than those receiving topical antibiotics alone.[1,7,10,11] Corneal epithelial cells cultured with amniotic membrane extract have decreased expression of matrix metalloproteinases 2 and 9, which may explain the documented inhibition of stromal melting associated with amniotic membrane extract use.[3] While promising, topical amniotic membrane extract has yet to be used in horses and further research is needed to determine whether equine corneal ulcer healing would benefit from the addition of topical amniotic membrane extract to the treatment protocol.

The purpose of this study was to determine whether adding a topical, commercially available amniotic membrane extract to antibiotic, antifungal and mydriatic medical treatment in horses with superficial corneal ulcers accelerated the healing rate compared with ulcers treated with medical therapy alone. Our hypothesis was that corneal ulcers treated with amniotic membrane extract would heal faster than those treated with only medical therapy. In addition, we sought to determine the rate of corneal re-epithelialisation with and without the addition of amniotic membrane extract to medical therapy.

2 | MATERIALS AND METHODS

2.1 | Experimental design

This study was designed as a masked, randomised, controlled experimental trial. All data collection, data input and image analysis were masked. Each horse served as its own control due to established, marked differences in individual corneal healing rates.[12,13] Bilateral ulcers were created concurrently to prevent the known, confounding effect of slower healing rates in the second eye when ulcers are created sequentially in horses.[14] Previous studies utilising concurrent, bilateral corneal wounding in rabbits, mice and cats have shown uncomplicated healing with minimal discomfort in both wounding and treatment phases.[8,9,13,15-17] This reduced the total number of horses needed for the study. The corneal ulcers were placed temporally to maintain clarity of the visual axis and involved only 6%-8% of the entire corneal surface area, thereby minimising the potential for visual disability. The study protocol included a statement for withdrawal if any animal developed unnecessary discomfort or complications. The likelihood of adverse events was considered low as the study used a commercially available product and prophylactic therapy to prevent infection.

2.2 | Sample size calculation

Thirty-six hours was estimated as the difference in healing time between control and treatment eyes with an estimated standard deviation of 36 hours. These time points were selected by extrapolating data from previous reports in which topical amniotic membrane extract decreased healing times by 9-48 hours in other species.[1,7,8,11] The sample size was calculated using this estimated difference, each horse serving as its own control, a 5% alpha error rate and at least 80% power. Greater than or equal to 9 horses was calculated as the appropriate sample size.

2.3 | Study population

In all, 10 healthy horses from the university's teaching and research herd were enrolled to account for potential drop out. Exclusion criteria included a history of ocular disease, presence of corneal disease (active lesions or fibrosis), adnexal abnormalities that could impair corneal healing or a temperament precluding frequent examination and medication administration.

2.4 | Surgical creation of corneal ulcers

A physical examination was performed and a packed cell volume and total protein determined. Horses were sedated at time 0 with butorphanol (0.01 mg/kg bwt IV) and detomidine hydrochloride (0.01 mg/kg bwt IV) with the addition of xylazine (0.3-0.6 mg/kg bwt IV) if needed. An auriculopalpebral nerve block was performed on both eyes (OU) with 1-2 mL of 2% lidocaine. A complete ophthalmic exam was performed including slit lamp biomicroscopy, application of fluorescein stain and indirect ophthalmoscopy with a 14D indirect lens.

Order of corneal ulceration was determined by coin toss. Tetracaine 0.5% ophthalmic solution (0.2 mL) was applied topically OU. Each eye and periocular area was prepared with 1:50 dilute betadine solution and sterile saline. A single surgeon (V.L.) created all ulcers. Using sterile technique, an 8 mm trephine was applied to the temporal paraxial cornea to delineate the ulcer border. A 1 mm

diamond burr (Algerbrush II Rust Ring remover, Alger Equipment Company) was used to remove the epithelium within the area delineated by the trephine (Figure 1). Fluorescein stain was applied to verify removal of all epithelium within the defined area. Subpalpebral lavage systems (SPL) (Mila, MILA International Inc) were placed in the inferior conjunctival fornix.[18] A protective mask, fly mask or visor was placed to prevent rubbing.

2.5 | Treatment

All topical treatments were administered through the SPLs and followed by 2 mL of air. Treatments were separated by at least 5 minutes. All treatments were initiated immediately after ulcer creation and continued until the corneal ulcer no longer retained fluorescein stain.

All eyes received 0.2 mL ofloxacin 0.3% ophthalmic solution four times daily, 0.2 mL of 1% silver sulfadiazine topical cream diluted 1:10 with sterile water four times daily and 0.2 mL of 1% atropine sulphate ophthalmic solution two times in the first 24 hours and then once daily. Ofloxacin was selected because of the minimal effect on epithelial healing rate and broad spectrum of activity against common aetiologies of equine bacterial keratitis including *Pseudomonas aeruginosa* and *Streptococcus zooepidemicus*.[19-21] Silver sulfadiazine 1% cream, diluted to a 0.1% solution to allow application through the SPL, was selected because of the broad fungicidal activity against filamentous fungal isolates from eyes of horses with keratomycosis.[22,23] Eyes in the treatment group received 0.2 mL amniotic membrane extract (EyeQ® Amniotic Eye Drops, EyeQ® Amniotic Eye Drops, Vetrix LLC) three times daily as recommended by the manufacturer. Eyes in the control group received 0.2 mL of the vehicle used to reconstitute amniotic membrane extract (manufacturer provided sterile saline, Vehicle used to reconstitute AME (saline, no active ingredient), Vetrix LLC) three times daily. These treatments were applied 5 minutes after administration of the antibiotic, antifungal and mydriatic medications listed above.

FIGURE 1 The diamond burr (Algerbrush II Rust Ring Remover with 1 mm burr) was used to remove superficial corneal epithelium within the area delineated by the 8mm trephine

The vehicle control and amniotic membrane extract were packaged identically and labelled as "X" or "Y" by the manufacturer. Before use, each bottle, "X" or "Y," was reconstituted with 5 mL of sterile saline (provided by Vetrix) and refrigerated. Each bottle, "X" or "Y," was shaken vigorously for 30 seconds prior to administration per manufacturer instructions to avoid settling. Selection of the eye in each horse that would receive "X" versus "Y" was determined by coin toss. As the solution's viscosity could inadvertently unmask the investigators, products "X" and "Y" were administered only by support staff. Horses received flunixin meglumine paste 1.1 mg/kg bwt by mouth every 12 hours until ulcers healed.

2.6 | Monitoring and measurement of re-epithelialisation

Horses were walked, lunged or turned out individually in pastures in view of other horses twice daily. Physical examinations were performed twice daily. Gastrointestinal motility based on borborygmi, and number and consistency of bowel movements were monitored twice daily. Packed cell volume and total protein were recorded every 48 hours. Mentation was graded as bright, alert and responsive; quiet, alert and responsive; or depressed. Comfort was measured by grading blepharospasm, conjunctival injection and chemosis as absent (0), mild (1), moderate (2) or severe (3). The pupil size, a measure of reflex uveitis, was graded as mydriatic (0), mid-sized (1) or miotic (2).

Slit lamp biomicroscopy and fluorescein stain were performed approximately every 12 hours following corneal wounding by the same masked observer. If needed, horses received xylazine (0.4-0.5 mg/kg bwt IV) to facilitate examination. Corneal ulcer size, shape and depth were recorded until healed. After fluorescein stain application, the eye was photographed with a macro lens (AF-S Micro Nikkor 105 mm, Nikon with D5300 camera, Nikon) and a fixed unit of measurement (Schirmer tear test strip) was included. Using these images, the surface area of dye uptake at each time point was calculated using ImageJ software as previously described (Figure 2).[24] Ulcers were considered healed when there was no fluorescein retention (Figure 3). The SPLs were then removed and the horse returned to the university herd.

2.7 | Data analysis

Time until complete healing for each eye was assessed for normality of distribution using the Shapiro-Wilk test. As the assumption of normality was not violated, healing time (hour) is presented as mean ± SD. For comparison of treatments, ulcer size was assessed. As the ulcer area size did not appear to follow a linear pattern over time, size was log-transformed based on a Box-Cox transformation

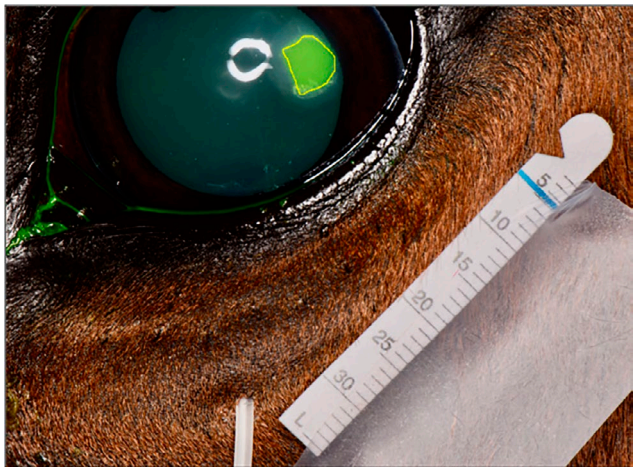

FIGURE 2 Left eye of horse 2 at 48 h post-ulceration. Ulcer surface area is demonstrated with a yellow circle using ImageJ software. The Schirmer tear test strip included in the image provided size reference when calculating ulcer surface area in mm^2

after adjustment by adding 0.01. A random coefficient regression model with repeated measures was used to compare corneal ulcer size/area, and hours until complete healing, between the treatment and control eyes nested within horse. The model can be written as

$$Y_{ijk} = \beta_0 + (\beta_1 + b_{1ij})\, t_{ijk} + \beta_2 trt_{ij1} + \beta_3 \left(trt_{ij1} \times t_{ijk}\right) + e_{ij} + h_i + \epsilon_{ijk},$$

where trt_{ij1}s are dummy variables/indicators for treatment 1, t_{ijk}s are the variables indicating the hours after surgery, e_{ij}s are random eye effects nested in horse and h_is are random horse effects. A spatial power structure was used to model the covariances among the errors due to unequally spaced time intervals for measurements. Correlation matrices indicated the nearer in time that measurements were made, the stronger the correlation between them. Model diagnostics included QQ plots and normality tests of scaled residuals.

Rate of re-epithelialisation was assessed using raw data irrespective of treatment assignment. After visualisation of the raw data, the linear model was divided into two separate linear parts due to the observed biphasic change in ulcer area. Slopes of the separate linear regression models were expressed with 95% confidence intervals. Significance level was set at $P < .05$. Data analysis was performed using commercially available software (SAS v.9.4, SAS Institute Inc.). After data analysis was complete, investigators were unmasked to treatment assignment.

3 | RESULTS

In all, 10 horses were included: 7 mares and 3 geldings. The average age was (mean ± SD) 16 ± 5 years, with a range of 5-25 years. Breeds included 4 Quarter Horses, 2 Standardbreds and one each of Mixed Breed Horse, Paint Horse, Thoroughbred and Tennessee Walking Horse. All treatments were well tolerated by all horses at all time points as evidenced by lack of blepharospasm and rubbing. Packed cell volume and total protein values, gastrointestinal motility and bowel movement number remained stable. All horses remained bright, alert and responsive.

Mild conjunctival injection (score 1) was noted at time of ulcer creation and persisted for 36-48 hours in all horses. One eye of one horse had mild conjunctival injection for 60 hours. Two horses had mild chemosis (score 1) immediately after ulcer creation that resolved within 24 hours, otherwise conjunctival injection and chemosis were absent (score 0). Blepharospasm was noted in one eye of one horse. The initial protective mask irritated the left eye for the first 12 hours. Blepharospasm resolved within 12 hours after the

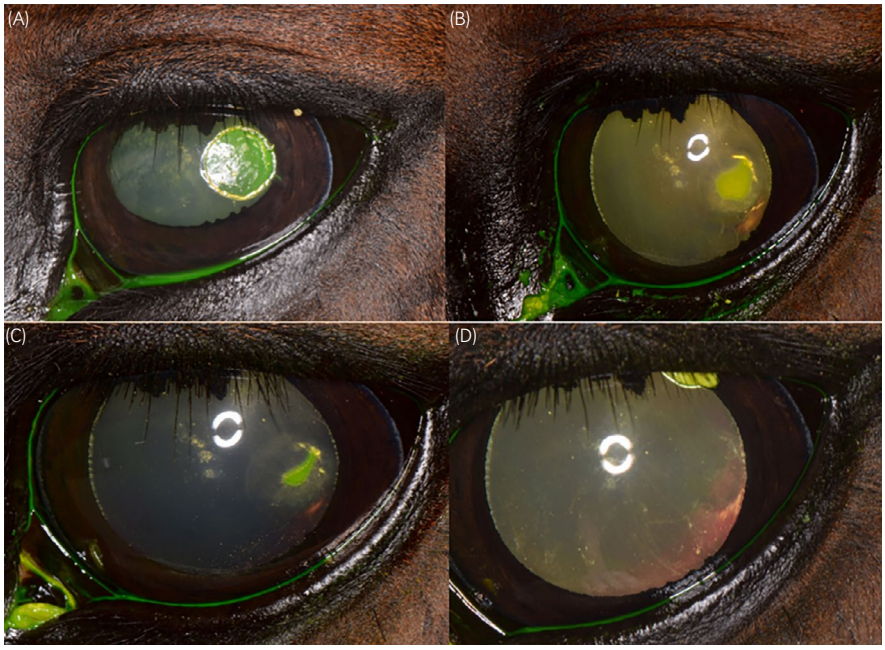

FIGURE 3 Left eye of Horse 4 treated with amniotic membrane extract. Corneal ulcers are highlighted by fluorescein stain. (A) 0 h post-ulceration; (B) 24 h post-ulceration; (C) 72 h post-ulceration; (D) 132 h post-ulceration (ulcer is healed; mucus is highlighted by fluorescein at the margin of the superior eyelid)

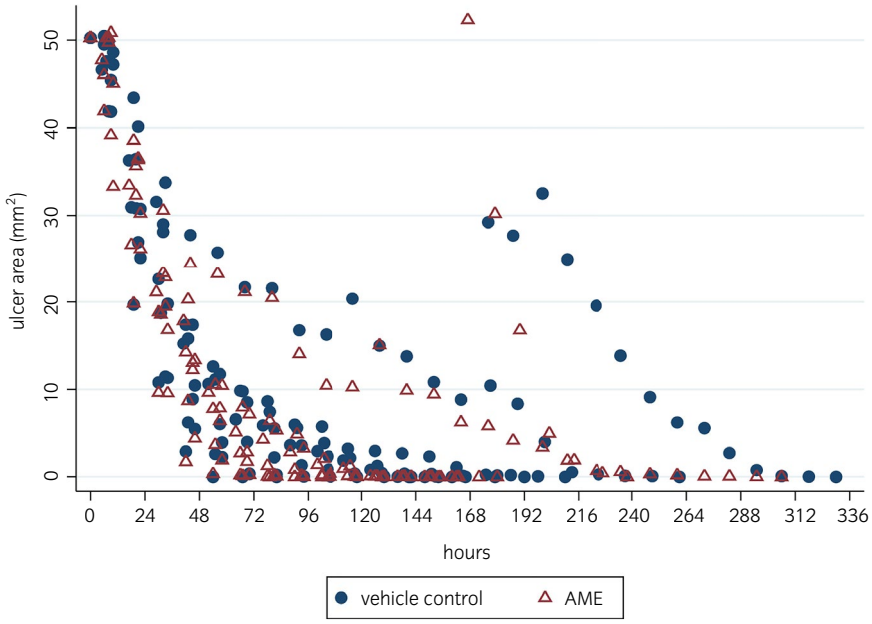

FIGURE 4 A scatterplot of ulcer surface area in mm^2 over time in hours demonstrating the size of ulcers in this study at each data collection point. Note that three eyes had a sudden increase in ulcer size between 168 and 180 h

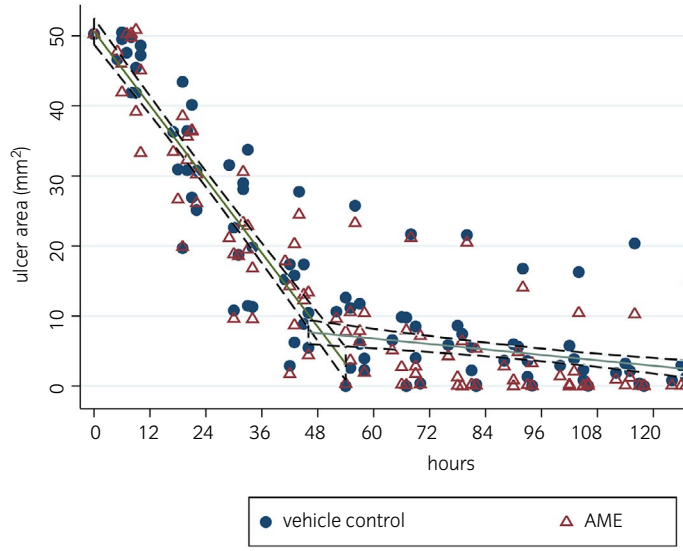

FIGURE 5 A scatterplot with best fit regression lines showing change in ulcer surface area over time. Vehicle control and amniotic membrane extract treatment groups both demonstrated biphasic healing, with an initial rapid phase of 0.88 mm^2/hr (95% CI: −0.81 to −0.94 mm^2/hr) followed by a slow phase of 0.07 mm^2/hr (95% CI: −0.04 to −0.09 mm^2/hr). Each distinct healing phase is demonstrated by the negative slope of each separate linear regression line demonstrated in this graph. There was no significant difference in time to healing between amniotic membrane extract and the vehicle control

protective mask was replaced with a visor. Substance "X" (control) was applied to 6 right eyes (OD) and 4 left eyes (OS). Substance "Y" (amniotic membrane extract) was applied to 4 OD and 6 OS.

All eyes healed within (mean ± SD) 165.2 ± 76.3 hours. Eyes treated with amniotic membrane extract healed in 162.7 ± 68.2 hours, whereas eyes treated with the control healed in 167.6 ± 87.4 hours. Data points for ulcer size over time in all horses are demonstrated in Figure 4.

Both eyes of a 17-year-old gelding healed slowly (336 hours OD [control] and 312 hours OS [amniotic membrane extract]). OS had loose epithelial edges at 180 hours that were debrided with a cotton tipped applicator. OD did not have loose epithelial edges. One eye each of 2 other horses (both control) required debridement of loose epithelium at 168 and 180 hours. After debridement, all of these eyes healed uneventfully. These four eyes had markedly delayed healing (range 240-336 hours). The average healing time without these four eyes was 135.5 ± 48.5 hours.

Although time was significantly associated with ulcer size/area ($P < .0001$), treatment group (amniotic membrane extract vs. control) was not significantly associated with size of ulcers over time ($P = .984$). Regardless of therapy, healing occurred in two distinct phases: an initial rapid phase with an average duration of 48-54 hours and healing rate (surface area reduction) of 0.88 mm^2/hr (95% CI: 0.81-0.94 mm^2/hr), and a second, slower phase with a variable duration and healing rate of 0.07 mm^2/hr (95% CI: 0.04-0.09 mm^2/hr) (Figure 5).

4 | DISCUSSION

In this study, adding a commercially available amniotic membrane extract topically to antibiotic, antifungal and mydriatic medical therapy for experimentally induced equine superficial corneal ulcers did

not significantly increase the rate of re-epithelialisation compared with medical therapy alone. This is in contrast to multiple studies in which human, rabbit and rodent superficial corneal ulcers healed faster when treated with amniotic membrane extract.[1,7,10,11]

The amniotic membrane extract source, dose and preparation method may contribute to healing effect. Most studies have used human amniotic membrane extract applied to human, rabbit and murine corneas.[1,7,10,11] Studies on equine corneal healing have all used equine amniotic membrane.[25-27] There are no studies examining the effect of amniotic membrane or amniotic membrane extract species source on the rate of corneal wound healing, nor whether allogeneic or heterogeneic amnion is superior. The commercial amniotic membrane extract used in the current study was prepared from bovine amnion. Freeze-dried bovine amniotic membrane applied to superficial canine corneal ulcers has been shown to significantly improve the rate of corneal healing compared with antibiotic, antifungal and mydriatic therapy alone.[28] No published studies exist regarding the effects of a bovine amniotic membrane extract on corneal wound healing.

The concentrations of bioactive compounds in amniotic membrane extract vary depending on preparation and storage methods with no standard or proven best method.[4,29] Some studies use fresh amniotic membrane homogenised in liquid nitrogen and then centrifuged, with the supernatant becoming the amniotic membrane extract.[4] Other amniotic membrane extract preparations with beneficial effects on corneal healing have been created from denuded, cryopreserved and/or dried amniotic membrane which was then pulverised, sonicated, homogenised and morselised alone or in combination.[4,7,10] Only one study has compared the effect of preparation method on extractable factors: pulverising amniotic membrane resulted in 20% more extractable factors compared with homogenisation and repeating centrifugation almost doubled the extracted factors.[30] The commercially available human products are processed using proprietary methods.[4,31] The product used in this study was created via cryomilling of bovine amniotic membrane, but further manufacturing details are proprietary. In this study, the amniotic membrane extract cell source and preparation method may have affected biomolecule availability and effect on corneal healing.

Only two previous publications have examined equine corneal wound healing. In one of these studies, 7 mm diameter keratectomies involving the anterior third of the corneal stroma healed in a median time of 11 days (range 5-37 days).[32] The wounds in the current study were larger (8 mm diameter), but healed faster (mean 6.5 days), likely because of the lack of stromal involvement.[33] In the second study, corneal epithelium was debrided to the limbus using a dulled scalpel blade.[14] The wounds healed in a biphasic manner with a rapid linear phase of 3 mm^2/h for the first 5-7 days, which then slowed. Rate of wound healing is determined by the equation:

$$R = \pi k D_o - 2\pi k^2 t$$

where R, healing rate in mm^2/h, k, velocity of epithelial cell migration in mm/h, D_o, initial wound diameter and t, time in hours.[13]

Larger D_o means faster healing rate in mm^2/h. In the current study, healing rate was reported differently because wounds did not heal in perfectly circular fashion. However, using the above equation and initial healing rate of 0.88 mm^2/h, initial wound diameter of 8 mm, and time of 24 hours when defects were relatively circular, velocity of epithelial cell migration was calculated to be 0.05 mm/h. This is very similar to the 0.048 mm/h rate of epithelial cell migration in horses previously reported and the 0.05-0.064 mm/h reported for rabbit corneal epithelial cell migration.[13,14,34]

After 48 hours in the current study and 5-7 days in a previous study, rate of healing slowed considerably.[14] A similar decrease in healing rate and delay in wound closure has also been reported in rabbits if healing takes longer than 48 hours.[13] In the equation above, a longer time period creates a larger value of ($-2\pi k^2 t$). Therefore, healing rate slows over time.[13] In a previous study on equine corneal healing, time to slowing was longer because of larger initial ulcer diameter.[14]

Similar to previous studies, the current study noted wide variation in healing times.[14,32] Similar findings have been noted in rabbits with marked variation between individuals in their healing rates.[13] These findings emphasise the importance of using each horse as its own control. One cannot entirely rule out that amniotic membrane extract treatment in one eye may have affected healing in the contralateral control eye due to an impact on a centrally mediated sympathetic ocular response which has been speculated as the cause of elevated matrix metalloproteinase 2 in the contralateral normal eye of horses with unilateral corneal ulcers.[35] However, an effect of amniotic membrane extract application on the contralateral, untreated eye has not been documented in previous studies. Furthermore, the rate of epithelial migration for all eyes in this study was the same as that in previous study of equine healing and very similar to that in other species, making a significant effect of amniotic membrane extract on the contralateral control eye unlikely.[14]

Many methods have been employed for corneal wounding: superficial keratectomies,[32,36] chemical debridement,[37,38] mechanical debridement with a blade[13,14] or rotating (diamond) burr debridements.[39,40-43] The rotating burr is preferred in mice, rats and rabbits.[44] Rotating (diamond) burr debridement was elected in this study due to decreased risk for recurrent erosions, erosions that form within weeks of the initial debridement.[39] Recurrent erosions were said to have occurred in two eyes of two horses in one previous study,[14] but no details were given regarding overall time to healing, number of recurrences or additional treatments required. One eye in another study[32] had a loose epithelial flap that occurred at day 17 post-ulceration, was debrided on day 18, recurred on day 21 and healed by day 25. Three eyes of three horses in the current study required cotton tip debridement of loose epithelial edges at 168-180 hours post-ulceration and then healed without further intervention. No eyes in the current study had a corneal ulcer recur after the experimental ulcer healed.

Diamond burr debridement has been associated with removal of the basement membrane within the wound centre as occurs in mice

or intermittently throughout the wound as occurs in dogs.[39,45] While presence or absence of the basement membrane does not alter the rate of epithelialisation, absent basement membrane and associated adhesion complexes may not fully reform for 5-6 weeks.[36] Within the current study, three eyes were noted to have loose epithelial edges at 167-177 hours post-ulceration. Central loss of the basement membrane may have prevented epithelial adhesion. However, even with an intact basement membrane, corneal epithelium does not reform hemidesmosomes which anchor epithelium to the basement membrane for 24-48 hours after healing and epithelium can be easily lifted off the corneal surface.[36] Perhaps horses rubbed despite wearing protective masks, fly masks or visors, disrupting fragile epithelium. Alternatively, shear forces from eyelids may have dislodged epithelium.[14] After debridement with a cotton tipped applicator, these wounds healed uneventfully.

Horses in the current study healed without developing corneal pigmentation or vascularisation. These results are in stark contrast to the significant inflammation, pigment migration and fibrosis noted in a previous study.[14] Polymorphonuclear cell infiltrates are important in stimulating corneal vascularisation.[46] Severity of corneal pigmentation correlates with degree of corneal inflammation.[47] In mouse models, use of a diamond burr decreases inflammatory cells recruitment to a corneal wound.[39] Decreased inflammation in corneas wounded with a diamond burr may explain lack of pigmentation and vascularisation in the current study.[39] Smaller ulcer size in this study may also contribute, as previous ulcers associated with these changes spanned the entirety of the equine cornea.[14] Furthermore, no horses in the current study developed corneal infections. In contrast, five of the eyes in a previous study became infected.[32] Four of these eyes did not receive antimicrobials, reinforcing the importance of adequate antimicrobial prophylaxis treatment for equine corneal ulcers.[32]

Corneal ulcers created by diamond burr debridement caused minimal discomfort. Diamond burr debridement has been associated with removal of the sub-basal nerve plexus at the wound site, with regrowth of this plexus being delayed by 4 weeks or more in mice.[39] This may explain why horses in this study were so comfortable. Excellent comfort makes this method an excellent model for studying equine corneal healing. In addition, overall lack of discomfort may also be partially attributed to preemptive and continued use of an oral NSAID and a topical cycloplegic in the form of atropine throughout the course of this study, similar to previous studies involving equine corneal wounding.[14,32]

4.1 | Conclusions

In this study, there was no significant difference in healing rate associated with adding a commercial amniotic membrane extract to antibiotic, antifungal and mydriatic therapy for experimentally induced superficial corneal ulcers in horses. A biphasic healing process was observed, with an initial rapid phase followed by a second slow phase. Further study is required to determine whether there is any beneficial effect of using amniotic membrane extract in infected or malacic equine corneal ulcers or difference in effect with other amniotic membrane extract formulations.

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CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

V. Lyons: primary responsibility for conception and design, acquisition of data, and analysis and interpretation of data and manuscript preparation. W. Townsend: primary responsibility for study conception and design and acquisition of data. G. Moore: primary responsibility of study design, analysis and interpretation of data. S. Liang: primary responsibility of data analysis and interpretation of data. All authors contributed to the manuscript and approved the final version.

ETHICAL ANIMAL RESEARCH

This study was approved by the Purdue University Animal Care and Use Committee (Protocol #1812001830, approved 1/11/2019). This study conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

INFORMED CONSENT

Not applicable.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13399.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Treatment of shoulder joint luxation with glenoid ostectomy in a miniature donkey

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CASE DESCRIPTION

A 13-year-old 128-kg miniature donkey gelding was evaluated for right forelimb lameness of 7 weeks' duration.

CLINICAL FINDINGS

Muscular atrophy of the infraspinatus and supraspinatus muscles over the right scapula with a palpable bony prominence over the point of the shoulder was evident. At the walk, the cranial phase of the stride was reduced with adduction of the distal aspect of the limb, dragging of the toe, and lameness (grade, 4/5). Lateral and craniocaudal radiographs of the right shoulder joint revealed lateral luxation of the humerus in relation to the scapula with bony proliferation and remodeling of the humeral head.

TREATMENT AND OUTCOME

Glenoid ostectomy was performed. Immediately after surgery, the donkey was able to intermittently stand squarely on the limb but maintained a reduced cranial phase of the stride at the walk. The donkey had no short-term complications and was discharged from the hospital 11 days after surgery. Following discharge, the donkey was confined to a box stall for 60 days, followed by a gradual increase in movement to full pasture turnout. The lameness continued to improve, and at 15 months after surgery the donkey was turned out in pasture and had mild lameness (grade, 3/5) at the trot. Mild muscular atrophy of the supraspinatus and infraspinatus muscles was present with no signs of pain on palpation or manipulation of the limb. Shoulder joint radiography at 15 months after surgery revealed remodeling of the glenoid cavity of the scapula and humeral head with formation of a pseudoarthrosis.

CLINICAL RELEVANCE

Glenoid ostectomy should be considered as an alternative to shoulder joint arthrodesis in small equids with shoulder joint luxation. Other indications for this procedure could include chronic osteoarthritis or fractures affecting the shoulder joint.

A 13-year-old 128-kg miniature donkey gelding was admitted with right forelimb lameness (grade, 4/5)[1] of 7 weeks' duration. No improvement had been observed for a period of 7 weeks since the time of injury. Trauma was suspected as the cause of lameness. Abnormal physical examination findings included muscular atrophy of the right supraspinatus and infraspinatus muscles, a palpable prominent greater tubercle of the humerus, and adduction of the distal aspect of the limb. At the walk, the cranial phase of the stride was reduced with dragging of the toe and partial weight-bearing on the limb. No neurologic deficits were observed. On the basis of physical examination findings, luxation of the shoulder joint was suspected.

With the donkey in a standing position, mediolateral and craniocaudal radiographic views of the right shoulder joint were obtained. Shoulder joint radiography revealed lateral luxation and bony remodeling of the right humeral head **(Figure 1)**. Surgical options, including closed reduction, open reduction, shoulder joint arthrodesis, and glenoid ostectomy, were discussed with the owner. On the basis of chronicity, we did not believe closed or open reduction would be possible, leaving shoulder joint arthrodesis and glenoid ostectomy as viable options. Glenoid ostectomy for chronic luxation or osteoarthritis of the shoulder has been previously reported for dogs.[2–6] These options were discussed with the owner, and they concluded that shoulder joint arthrodesis was not a financially viable option. Therefore, the owner elected for glenoid ostectomy if either open or closed reduction of the shoulder joint luxation was not possible.

Penicillin G potassium (22,000 U/kg, IV), gentamicin sulfate (6.6 mg/kg, IV), phenylbutazone (4.4 mg/kg, IV), and a tetanus toxoid vaccine (IM) were administered prior to anesthetic induction. Sedation prior to induction of general anesthesia consisted of administration of xylazine hydrochloride (0.78 mg/kg, IV) and butorphanol tartrate (0.04 mg/kg, IV). Anesthetic induction was achieved by administration of midazolam (0.12 mg/kg, IV), ketamine (2.3 mg/kg, IV), and guaifenesin (50 mg/kg, IV). General anesthesia was maintained with isoflurane (2% to 3%) in oxygen. The donkey

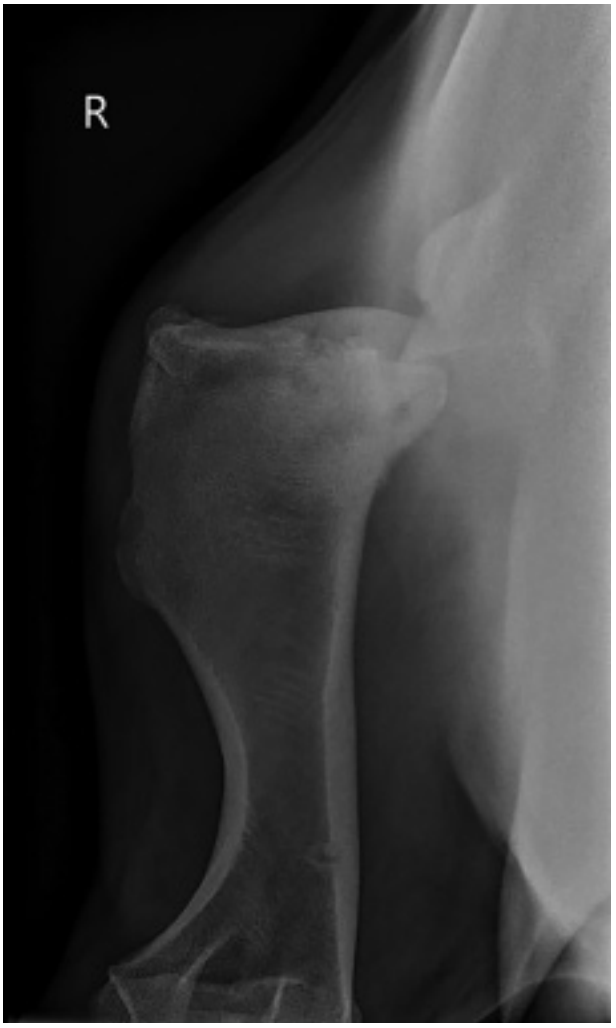

Figure 1—Preoperative craniocaudal radiographic view of the right shoulder joint with lateral luxation of the humerus in relation to the scapula with bony proliferation and remodeling of the humeral head.

was positioned in left lateral recumbency. Closed reduction was attempted unsuccessfully via traction with a come-along winch prior to continuing with open reduction and glenoid ostectomy. The hair overlying the surgical site was clipped and the area was aseptically prepped and draped, including draping of the distal aspect of the limb to allow manipulation of the limb.

A 20-cm-long craniolateral skin incision centered over the shoulder joint was made followed by sharp dissection through the subcutaneous layer and omotransversarius muscle with cranial retraction of the brachiocephalicus muscle. Tenotomies of the insertional tendons of the supraspinatus and biceps brachii muscles were performed, and the joint capsule was incised parallel to the joint surface. At the site of luxation, cartilage and bone erosion of the lateral aspect of the glenoid cavity of the scapula and the medial aspect of the humeral head was observed. Because of excessive fibrosis and the chronicity of the luxation, open reduction was not possible. An oscillating saw and osteotome were used to remove the glenoid cavity with a transverse ostectomy through the scapular neck, avoiding the suprascapular nerve. The joint capsule was sutured closed, and the tendons of the supraspinatus and biceps brachii muscles were reattached to their insertions with inverted cruciate and simple interrupted patterns with size-1 polyglactin 910. The omotransversarius muscle incision was closed in the same fashion. The subcutaneous layer was closed with a simple continuous pattern of size-0 polyglactin 910. Sterile saline (0.9% NaCl) solution containing gentamicin sulfate was used for incisional lavage, and stainless steel skin staples were used to close the skin. A stent bandage and iodine-impregnated incision drape were secured over the wound. Total surgical time was 1 hour and 14 minutes. Xylazine hydrochloride (0.23 mg/kg, IV) was given once in the recovery stall. Recovery from anesthesia was manually assisted with 2 handlers and was without complication.

After surgery, the donkey was able to intermittently stand, with the right forelimb underneath its body, and bear weight on the limb in the stall. At the walk, the donkey maintained a reduced cranial phase of the stride with mild improvement, compared with before surgery. The donkey received potassium G penicillin (22,000 IU/kg, IV, q 6 h), gentamicin sulfate (6.6 mg/kg, IV, q 24 h), and phenylbutazone (2.2 mg/kg, IV, q 12 h) for 5 days after surgery. Following discontinuation of IV administration of antimicrobials, trimethoprim sulfamethoxazole (30 mg/kg, PO, q 12 h) was administered for 7 days. The stent bandage was removed 3 days after surgery. Box stall rest was continued for the donkey for 2 months after hospital discharge before a gradual increase in exercise to full pasture turnout.

At 15 months after hospital discharge, a follow-up examination was performed at the teaching hospital. Supraspinatus and infraspinatus muscular atrophy was present but improved, compared with before surgery. There was no pain on palpation or on flexion and extension of the limb, and range of motion was comparable to the other forelimb. No lameness at the walk was observed; however, at a trot, a consistent but mild lameness (grade, 3/5)[1] was present with a subtle reduced cranial phase of the stride. Radiographs revealed bony remodeling of the glenoid cavity of the scapula and humeral head with formation of a pseudoarthrosis **(Figure 2)**. The long-term outcome was considered excellent on the basis of absence of pain associated with the shoulder, functional use of the limb with improvement in lameness, and no postoperative complications. The owner was satisfied with the outcome and reported that improvement in lameness after surgery was gradual, with the most improvement observed in the 6 months prior to reexamination.

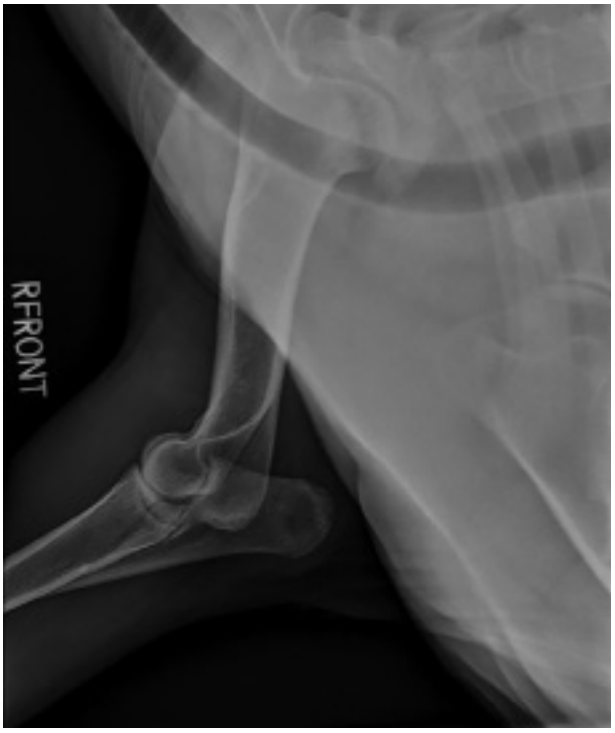

Figure 2—Fifteen-month postoperative mediolateral radiographic view of the right shoulder joint with chronic bony remodeling of the glenoid cavity of the scapula and humeral head with formation of a pseudoarthrosis.

Discussion

Shoulder joint luxation in large animals is an uncommon orthopedic condition that can be characterized by non– to minimally weight-bearing lameness, gait alterations including reduced range of motion (reduced cranial phase of the stride) or a tendency to drag the limb, and pain upon palpation and manipulation of the shoulder.[7–9] Luxation of the shoulder is obvious upon physical examination because the greater tubercle of the humerus is displaced laterally and, with chronicity, there is disuse muscular atrophy overlying the shoulder joint. Sudden trauma is the most common etiology and is often assumed in cases where a cause is not identified.[7] Miniature horses and ponies are overrepresented and may be predisposed to shoulder dysplasia as, at least in Shetland ponies, a shallower glenoid cavity leading to instability has been reported.[10–12] The prognosis for this problem is poor without treatment and worse with concurrent fractures and osteoarthritis.[7] Although closed reduction has been successful in acute cases, the success rate is lower, compared with that of open reduction.[7,13–17] Many options for intervention have been attempted, such as open reduction and internal stabilization with biceps brachii tendon transposition, joint capsule imbrication, and lateral tension band sutures.[7,18–20]

Shoulder joint arthrodesis with internal fixation has been reported for small and miniature horses.[18,19,21] Variations in the technique have been described and can include transarticular Steinman pins in addition to a dynamic compression plate with transarticular lag screws.[18,19,21] This technique is associated with the increased cost of internal fixation and has the risk of complications such as fractures, implant failure, and surgical site infection.[19,21] For shoulder joint abnormalities not amenable to restoration of joint function, shoulder arthrodesis yields good to excellent outcomes but can be associated with complication rates as high as 50%.[22–24] In dogs and cats, glenoid ostectomy with or without humeral head excision has been described as an alternative to shoulder joint arthrodesis.[2–6] The pseudoarthrosis that forms is characterized by sclerosis of the ends of bone and creation of fibrocartilage and a fibrous capsule, which is caused by motion at the site of nonunion where the ostectomy was performed.[25] Glenoid ostectomy offers advantages over arthrodesis of simplicity, maintenance of some degree of shoulder mobility, lower surgical costs, and no implant-associated complications because orthopedic implants are not used. In small animals, glenoid ostectomy has favorable, long-term results with return to pain-free function.[2,3]

To the authors' knowledge, the present case report is the first description of a shoulder joint luxation in an equid treated with glenoid ostectomy. This technique was chosen because shoulder joint arthrodesis was not an option for the owner. Glenoid ostectomy resulted in an acceptable postoperative outcome on the basis of patient comfort and function. On the basis of our experience with this technique, we believe glenoid ostectomy should be considered as a viable and affordable alternative to shoulder arthrodesis in small-breed equids. This technique required minimal postoperative care, no orthopedic implants, decreased anesthetic and operative times, and decreased soft tissue dissection during surgery and was not associated with any major complications. In this case, postoperative rehabilitation involved confinement for 2 months prior to turnout into larger areas to encourage walking and movement followed by unrestricted pasture turnout.

In regard to patient size, we are not able to make recommendations for how the technique would work for full-size horses, but it would be useful in small ponies and miniature horses and donkeys. To our knowledge there are no reports of substantial anatomic differences between donkey and horse anatomy that would affect the surgical procedure or outcome. We have concerns for contralateral limb laminitis in larger-sized horses because our patient was not immediately fully weight-bearing on the affected forelimb after surgery. Although we only have experience with a single case, glenoid ostectomy could be considered as an alternative to arthrodesis in small equids with chronic osteoarthritis or fractures affecting the shoulder joint. In summary, glenoid ostectomy is an acceptable alternative to shoulder joint

arthrodesis in small equids with chronic luxation of the shoulder joint. It was associated with lower cost (compared with arthrodesis), a low rate of complications, and an acceptable postoperative outcome.

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