PRIMER

Progress in laminitis research, a primer for: Distribution of technetium-99m PEG-liposomes during oligofructose induced laminitis development in horses

Tami Rahkola
Department of Biology, University of Minnesota Duluth

Purpose

This primer aims to provide sufficient background to explain "Distribution of technetium-99m PEG-liposomes during oligofructose induced laminitis development in horses," and subsequently assist in understanding the consequence of that paper on the field of laminitis research. Underwood, Pollitt, Metselaar, Laverman, van Bloois, van den Hoven, Storm, and van Eps are leaders within this field of research. Their experiment, being discussed in this primer, aimed to investigate the accumulation and biodistribution of liposomes during an oligofructose experimental model of laminitis in equines. Radio-labeling the liposomes allowed these researchers to identify that liposomes readily accumulate in the lamellar tissue of laminitic horses. Liposomes have been used for drug delivery in other disease models, due to their ability to carry aqueous solutions in their inner compartment. Knowing that they accumulate in the lamellar tissue during laminitis development indicates that they may be useful for targeted drug delivery to the lamellar tissue. This could provide the breakthrough in finding a clinical application for pharmaceutical intervention of laminitis.

INTRODUCTION

Laminitis is a painful disease that affects equines of all breeds, all ages, and both genders. It also impacts horse owners and veterinarians, contributing to $102 million in treatment costs per year (Lameness 2000). Laminitis occurs when active tissue, called lamellar tissue, inside the hoof swells. The lamellar tissue is an integral part of a complex arrangement of tissues that hold the hoof wall to the distal phalanx (Katz et al. 2012; Pollitt 2004, 2010). The **distal phalanx** is the lowest bone within the hoof (Figure 1A).

Distal phalanx: Commonly called the coffin bone, resides within the hoof. See Figure 1

In minor laminitis cases, the horse will experience discomfort and may limp while walking. In more serious cases, the distal phalanx may separate from the lamellar tissue. This causes the bone to sink and the hoof wall to rotate away from its natural position (Engiles et al. 2015; Holl et al. 2015; Li et al. 2015). In the most severe cases, the bone may sink through the sole of the hoof (Laskoski et al. 2016) and cause the demise of the horse (Katz et al. 2012; Gardner et al. 2015). Laminitis has no cure, and there are three main schools of thought on what causes it: supporting limb, endocrinopathic, and sepsis-related. These are briefly detailed within this primer.

Corresponding Author:
Tami Rahkola
rahko032@d.umn.edu

This primer will first provide background information on the complex elements that contribute to sepsis-related laminitis, and then this primer will provide an explanation of Underwood et al. (2015b)'s research article, "Distribution of technetium-99m PEG-liposomes during oligofructose induced laminitis development in horses." The purpose for this two-part layout is to assist in the reader's understanding of the exciting implications from the Underwood et al. (2015b) study.

BACKGROUND

THE HOOF

Keratin: A fibrous structural protein

The hoof is at the base of the leg, and the four hooves collectively support the weight of the entire horse. The hoof wall is a hard **keratin** material that continually grows throughout the life of the horse from the coronet band (Figure 1A), also protecting the inner hoof structures (Pollitt 2004, 2010). The inside layer of the hoof wall begins a complex collection of support tissue, proteins, enzyme/protein transport, and epidermal tissues that work to make the hoof an integral part of the whole circulatory system and to provide support for the entire body (Pollitt 2004, 2010; Katz et al. 2012). The epidermal cells of the lamellar tissue within this complex are constantly being stressed by compaction and flexion of the outer hoof wall (see primary lamellar, Figure 1B), so the health of these lamellar epidermal cells is central to a healthy hoof (Pollitt 2004).

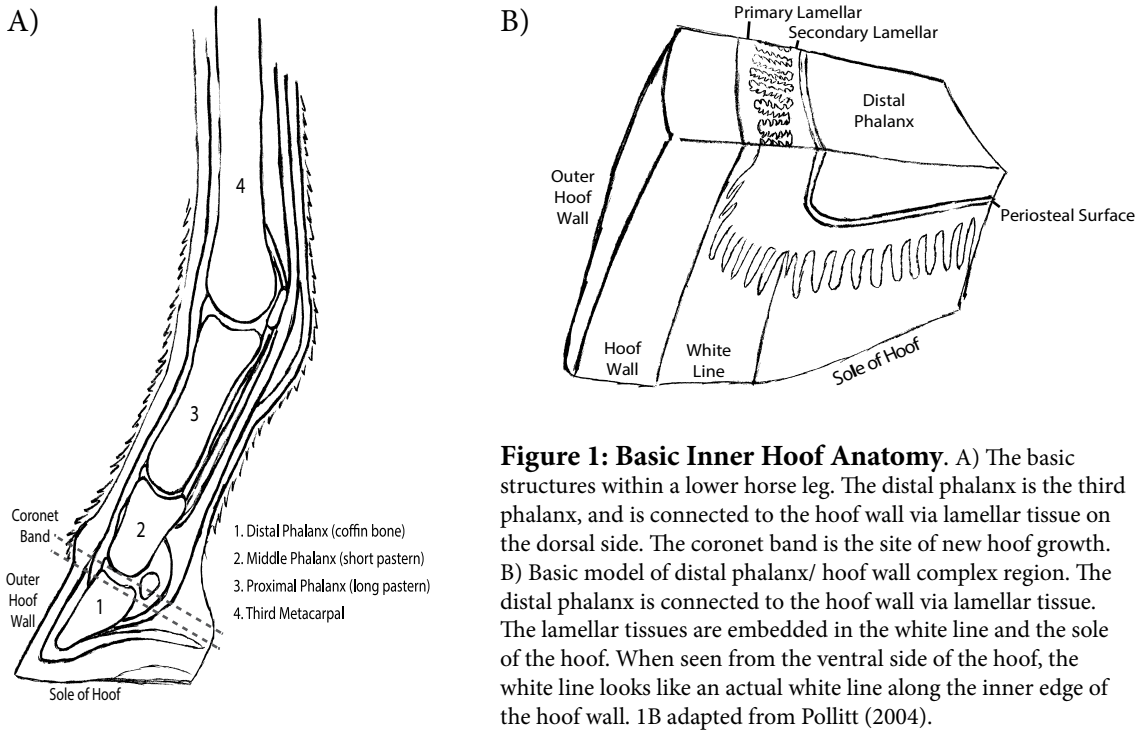

Figure 1: Basic Inner Hoof Anatomy. A) The basic structures within a lower horse leg. The distal phalanx is the third phalanx, and is connected to the hoof wall via lamellar tissue on the dorsal side. The coronet band is the site of new hoof growth. B) Basic model of distal phalanx/ hoof wall complex region. The distal phalanx is connected to the hoof wall via lamellar tissue. The lamellar tissues are embedded in the white line and the sole of the hoof. When seen from the ventral side of the hoof, the white line looks like an actual white line along the inner edge of the hoof wall. 1B adapted from Pollitt (2004).

LAMINITIS

Laminitis is an inflammation of the lamellar tissue that connects the hoof wall to the distal phalanx. The pathogenesis of laminitis is complex and not well understood. The schools of thought on what causes this disease vary, though three main theories prevail: endocrinopathic, supporting limb, and sepsis-related. Endocrinopathic laminitis is linked to obesity and Equine Metabolic Syndrome, which is similar to Type 2 diabetes in humans (Katz et al. 2012; Kaczmarek et al. 2016). Supporting limb laminitis is defined as limited movement will lead to decreased blood flow and to an eventual disruption of the lamellar/distal phalanx complex (Katz et al. 2012; Medina-Torres et al. 2014). Sepsis-related laminitis is similar to sepsis-related diseases in humans, in that sepsis is systemic inflammation in response to infection (Cawcutt and Peters 2014).

Laminitis has similar outcomes to that of sepsis-related organ failure and of systemic inflammatory response syndrome (SIRS) in humans (Belknap and Black 2012; Tadros et al. 2012; Cawcutt and Peters 2014; Underwood et al. 2015a).

Laminitis, in all forms, has expressions of inflammation, enzyme dysregulation, and leukocytosis (van Eps and Pollitt 2006; Katz 2012; Tadros et al. 2012; Underwood et al. 2015a; Laskoski et al. 2016). Some forms of laminitis also show the same process of endothelial inflammation, leukocyte infiltration, and may lead to an increase in enzymes related to inflammation, as sepsis in humans does (Underwood et al. 2015b).

The pathogenesis of endocrinopathic and sepsis-related laminitis is thought to progress as follows: a high influx of fructose decreases hind-gut pH, causing the gram-negative bacteria to die. This increases lipopolysaccharide concentration, which in turn activates leukocytes (Li et al. 2015). This leukocyte activation, which is a common theme in each form of laminitis, creates inflammation, which in turn leads to the disruption of the hoof wall/lamellar tissue/distal phalanx adhesion complex (Gardner et al. 2015; Katz et al. 2012; Li et al. 2015). Leukocyte activation also plays a role in the activation of matrix metalloproteinases (MMPs).

MATRIX METALLOPROTEINASES

The activation of MMPs is linked to tissue inflammation within the sepsis-related laminitis model. These MMP enzymes play a role in the breakdown of extracellular matrix (ECM) proteins (Pollitt 2004; Katz and Bailey 2012; Visser and Pollitt 2012; Li et al. 2015; Underwood et al. 2015a; Visser and Pollitt 2012; Kaczmarek et al. 2016). Two MMPs have been actively studied in laminitis: MMP-2 and MMP-9 (Li et al. 2015; Kaczmarek et al. 2016). These two are part of the gelatinase family of MMPs, which are located in **neutrophil** cells. This family is known to play a role in vascular permeability and in the degradation of collagen and the ECM. (Katz et al. 2012; Li et al. 2015; Kaczmarek et al. 2016). The uncontrolled breakdown of ECM proteins may lead to inflammation and subsequent laminitis (Visser and Pollitt 2012). ECM proteins in the hoof wall are continually remodeled through the inhibition/activation of MMPs; careful MMP regulation is part of what allows the distal phalanx to remain attached to the hoof wall as the hoof wall moves (Pollitt 2004). Movement can be from compression/flexion as the horse walks (Wattle and Pollitt 2004), or it can be from movement as the new hoof wall grows down from the coronet band (Pollitt 2004).

Neutrophil: Small white blood cell that is part of the immune system

OLIGOFRUCTOSE

The role of MMPs in laminitis pathogenesis is often investigated using the oligofructose (OF) model of laminitis induction. OF is the most current method for laboratory study of laminitis development because it consistently produces laminitis (Jian et al. 2015). A small amount of OF consumption (by horses) does not negatively impact them, but consuming large amounts of OF produces laminitis. OF is an **inulin**-like polymer of fructose, which is commonly extracted from chicory (van Eps and Pollitt 2006; Tadros et al. 2013). This fructose is similar to the fructans in pasture grass. Grass fructans can aggregate in high concentration in the stems (van Eps and Pollitt 2006), which horses eat. An overload of carbohydrates, such as too much high-fructose grass, is known to cause lamellar inflammation on short-time scales, with the potential to cause laminitis if the overload is continued (Wattle and Pollitt 2004; Medina-Torres et al. 2015; Kaczmarek et al. 2016). Because OF induces laminitis, due to its similar properties to carbohydrates, Underwood et al. (2015b) used OF in their study in order to initiate laminitis.

Inulin: Starchy carbohydrate found in many plants. Not to be confused with the hormone *insulin*

LIPOSOMES

Using OF-induced inflammation, Underwood et al. (2015b) investigated a potential means of blocking the inflammation with liposomes. Liposomes are nanoparticles with a **phospholipid**

Phospholipid: Two fatty acid chains attached to a glycerol head. See Figure 2

bi-layer structure (Figure 2). This structure allows them to penetrate the hydrophobic barriers of membranes that other small particles cannot pass through. Liposome structure has both hydrophobic and hydrophilic components, which allows them to carry both hydrophobic and hydrophilic particles. Liposomes can also be manufactured in the lab for the specific requirements of an experiment, such as size, composition, surface characteristics, and charge (Underwood and van Eps 2012). Some laboratory studies have investigated liposome use for targeted drug delivery in difficult diseases such as cancer (Underwood and van Eps 2012).

Liposomes have also been used in medicine for topical, intravenous, and intramuscular applications (Underwood and van Eps 2012), but they are prone to reticuloendothelial system uptake. This means they can trigger the immune system defense (Arulsudar et al. 2004), which can cause severe hypersensitive reactions (Szebeni et al. 2007). Coating the liposome with an outer substance can reduce immune system recognition, the subsequent reactions, and can increase liposome circulation time. A common coating used is polyethylene glycol (PEG), due to its hydrophilic nature and proven ability to increase liposome blood circulation time (Oyen et al. 1996; Laverman et al. 2000; Arulsudar et al. 2004; Underwood and van Eps 2012; Underwood et al. 2012). Liposomes were deemed safe for use in equine lamellar studies at slow infusion rates (Underwood et al. 2012).

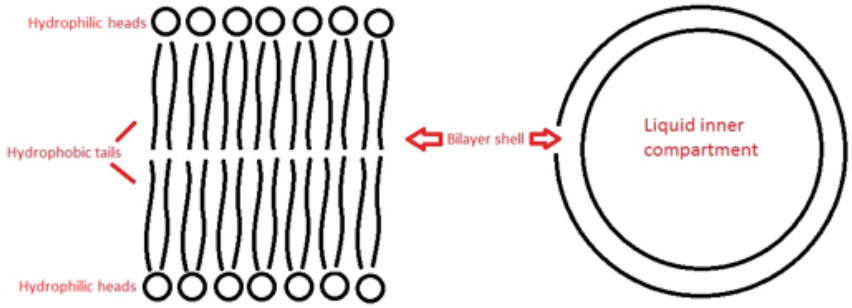

Figure 2: A Liposome. These contain a liquid inner compartment which is surrounded by a bi-layer shell. The bi-layer shell is composed of phospholipids which have a hydrophilic (water loving) outside layer and a hydrophobic (water fearing) inside tail layer.

NUCLEAR RADIOLOGY

Tagging a liposome with a radioactive tracer can offer insight to how the liposomes disperse in a study subject. Nuclear radiology accomplishes this by attaching a radioactive particle to a component of interest, such as insertion into the aqueous part of a lipid particle (a liposome) (Oyen et al. 1996; Arulsudar et al. 2004). Nuclear radiology has been employed in medical research with cancer and inflammatory diseases (Oyen et al. 1996). Radioactive tracers are made of radionuclides, or the nuclei, of unstable isotopes. These isotopes attempt to stabilize by emitting radiation (charged particles) during their decay (Ziessman et al. 2013), which allows them to be traced with scintigraphic cameras as they move throughout a system. Isotopes are chemical elements which consist of a different number of neutrons. Elements decay at a rate specific to themselves, known as their half-life. This half-life is the time it takes for one-half of the original amount to decay. Understanding isotopic half-lives is essential to choosing the best isotope for a specific study. Medical studies use a light radioactive element with a short half-life, which does not release beta emissions. Using isotopes without beta emissions reduces the potential toxicity to the both subject and researcher (Ziessman et al. 2013).

The sections listed above provide the necessary background for the full comprehension of the Underwood et al. (2015b) paper, which is discussed below.

"Distribution of technetium-99m PEG-liposomes during oligofructose induced laminitis development in horses"[i]

Using the knowledge discussed above, Underwood et al. (2015b) applied these techniques to investigate if liposomes would accumulate in lamellar tissues during laminitis development. If the results of their investigation showed that liposomes did accumulate in the tissues, then liposomes could possibly be approached as a drug delivery method for laminitis prevention.

The authors of Distribution are with, or connected to, the Australian Equine Laminitis Research Unit (AELRU) at the University of Queensland, Australia. Since the 1990s, the AELRU has been a leader in the field of laminitis research, with two authors of "Distribution" (Pollitt and van Eps) having been inducted into the International Equine Veterinary Hall of Fame in Kentucky, U.S.A. The AELRU works closely with The University of Pennsylvania, Ohio State University, and others in their pursuit of laminitis knowledge. Another of AELRU's 2015 papers won a prestigious award for influencing or improving clinical practice (Australian 2015). "Distribution" is the first paper to explore the dispersal of liposomes in a sepsis-related animal model (Underwood et al. 2015b).

In "Distribution," Underwood et al. (2015b) used radio-labeled liposomes which enabled them to determine the dispersion of the liposomes within the horse study subjects. These subjects were given oligofructose (OF) to instigate laminitis. If the radio-labeled liposomes accumulated in the lamellar tissue of the laminitic horses, then future investigations could use liposomes to deliver MMP-inhibiting pharmaceuticals.

EQUINE SUBJECTS

The "Distribution" project was done under approval of the University of Queensland Animal Ethics Committee, and complied with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (Underwood et al. 2015b).

Standardbred: American horse breed known for its trot

Gelding: A male horse that is no longer intact

Nasogastric tube: Tube that goes through the nose into the stomach

The project in "Distribution" used ten **Standardbred geldings** with healthy feet; these horses were obtained and cared for, for one month prior to the start of the study. For the experiment itself, the horses were kept in lead-lined stalls (to mitigate the effects of the radiation used in the experiment). Six of these subjects were chosen for the oligofructose-induced laminitis group (OFG), and the other four were maintained as the control group. A **nasogastric tube** was used to administer either plain water (control group), or 10 grams of oligofructose (OFG) for every kilogram of body weight, as detailed in van Eps and Pollitt (2006). The horses in "Distribution" were monitored throughout the study for heart rate, respiratory rate, temperature, and lameness. Once an Obel grade 2 lameness was evident in the laminitis group (Table 1), one dose of phenylbutazone (a common equine pain medication) was given intravenously.

Table 1: Obel lameness scoring chart. Used to assess lameness in horses. Adapted from Jiang et al. (2015).

Grade	Symptoms
1	Continual weight shifting when standing, no lameness at a walk, short gait when trotting
2	Altered gait at a walk, horse easily yields hoof for assessor
3	Altered gait at walk and trot, will not yield hoof to assessor
4	Excessive lameness, will only walk if forced

LIPOSOME PREPARATION, RADIO-LABELING, AND ADMINISTRATION

The liposomes used in "Distribution" were made from a phospholipid, Dipalmitoyl phosphatidylcholine (DPPC), and coated with polyethylene glycol, PEG-(2000)-distearoyl phosphatidylethanolamine (PEG-(2000)-DSPE). The liposomes were made using the film hydration method (Underwood et al. 2012; Underwood et al. 2015b).

Generally this method is conducted by combining phospholipids, polyethylene glycol, and a chloroform/methanol mixture. This combination would be spun at high rates and then flushed with nitrogen to remove any solvent present, leaving only a very dry film of lipids (SL Stevenson, personal communication, February 12, 2016). The lipids would then be added to a reducing agent (GS-H in "Distribution") to prevent clumping. A buffer (HEPES in "Distribution") is then added to maintain the required pH. The lipid/GS-H/buffer solution would then undergo dialysis to remove empty liposomes and un-entrapped GS-H, leaving only GS-H filled liposomes. Combining the lipid/GS-H/buffer in solution would also result in large multi-layer vesicles which need to be broken down to the correct sizes required for the experiment at hand (SL Stevenson, personal communication, February 12, 2016).

In "Distribution," this sizing was done using filters with sequentially smaller pore sizes, until the average size of liposomes was 85 nm. Underwood et al. (2015b) then labeled the finished liposomes with the radioactive tracer: 99mTc-hexamethylpropylene-amine-oxime (HMPAO). This tracer is commonly used to determine regional physiological changes in blood flow, and it is easily converted to a long-lasting hydrophilic state (Isaka et al. 2000). When the tracer is added to the liposomes containing the GS-H, the tracer will enter the liposome. The GS-H then acts as a reducer on the oxide bond between the 99mTc and the HMPAO (SL Stevenson, personal communication, February 12, 2016). The hydrophilic 99mTc is then trapped within the aqueous core of the liposomes. Un-encapsulated 99mTc-HMPAO would then be removed via gel filtration (Underwood et al. 2012). In the "Distribution" study, a calibrator was used to discern the labeling efficiency by measuring the activity of the 99mTc-HMPAO that was bound to the liposomes as well as that which was not bound to liposomes.

Upon completion of radio-labeling the prepared liposomes, all subjects in the study received 300 μmol of the radioactively labeled liposomes (RLL) intravenously. An additional 5.5 μmol of non-labeled liposomes were added to ensure that the total liposome concentration was above the level of rapid clearance, as liposomes can be endocytosed while in circulation (Laverman et al. 2000). The subjects in the "Distribution" OFG were divided into three sub-sections, and each section received the RLL, either 0, 12, or 18 hours after OF dosing. To reduce the risk of hypersensitivity reactions common in liposome administration (Szebeni et al. 2007), Underwood et al. (2015b) state that the dose was given slowly; heart, respiratory rates, and temperature were monitored for one hour, beginning at the start of the infusion.

POST-ADMINISTRATION EVALUATION

The "Distribution" study used a scintillation camera at 1, 6, and 12 hours after RLL infusion was begun, to monitor the distribution of the RLL in every subject. Additional scans of the 0-hour OFG were done at 18 and 24 hours post-infusion, and the final 12 hours of the 0-hour OFG were also used for analysis (Underwood et al. 2015b).

Blinded observer: A person who does not know the specifics of the treatment being done

Images from every subject included the dorsal and lateral sides of both forefeet, as well as of the hoof, fetlock, metacarpus, and the area between the hoof/metacarpus and fetlock/metacarpus. Specialized software was used to display and analyze the images by a **blinded observer**, and the measurements were done three times. The count density of radioactivity was assessed, and adjustments for decay and the dosage given were made.

Technetium: Chemical element with no stable isotopes (all forms are radioactive)

Underwood et al. (2015b) collected blood samples from the subjects at 0, 6, and 12 hours post infusion, and recorded the radioactivity of each sample via shielded well scintillation gamma counter. They also performed the same adjustments for decay and dose. Using an Excel add-on, they estimated the half-life of the radio-labeled liposomes. The radioactive decay of **technetium** alone is known to be around 6 hours (Ziessman et al. 2016). Therefore, Underwood et al. (2015b) calculated the radio-labeled liposomes (RLL) to understand changes to the decay rate when liposomes are added. Following the last radioactivity assessments of each group, the subjects were tested for lameness via the Obel system and were then euthanized.

Tissue samples were immediately taken from areas of interest, including forelimb lamellar tissue. This collected tissue was assessed to discern the amount of the radioactive liposomes present, again via shielded well scintillation gamma counter. Simultaneously, Underwood et al (2015b) assessed a dose of the original RLL that they had kept to assist in correcting for the known physical decay of the technetium within the RLL. Finally, dorsal lamellar sections were obtained, fixed via paraffin embedding, and a blinded observer assessed the subsequent laminitis pathology. All data was analyzed via statistical software.

"DISTRIBUTION" RESULTS

Each horse in the OFG had developed the outward symptoms expected from oligofructose-induced laminitis (Szebeni et al. 2007; Table 1), though the liposome infusion produced no hypersensitivity reactions (Underwood et al. 2015b). The average radio-labeled liposome (RLL) concentration, activity, and radioactive decay, along with the hoof wall surface temperature were consistent between the OFG and control groups.

The radioactivity within specific regions differed between the two groups. The OFG showed an increase in tissue radioactivity, especially in the dorsal hoof, lateral hoof, fetlock, lateral hoof/metacarpal, and fetlock/metacarpal. Conversely, the OFG showed an overall decrease in blood RA, to the point of becoming lower than the control group at 12 hours. The control group showed a radioactivity decrease in the dorsal hoof, lateral hoof, fetlock, and metacarpal regions over time. Most importantly, the OFG had higher RLL in their lamellar tissue than did the control horses; the highest levels were seen in the group receiving the liposomes 18 hours after OF induction. This level was over quadruple (4.8 fold increase) that of the control horses.

These results from "Distribution" support Underwood et al. (2012)'s findings that liposomes do appear in lamellar tissue during oligofructose induced laminitis. The concentration of liposomes in the OFG lamellar tissue at 12 hours supports the idea that liposome-delivered pharmaceuticals could maintain prolonged concentrations in lamellar tissue. Similarly, as no hypersensitivity reactions were observed with liposome administration, this study further supports the benefits of slow intravenous liposome delivery of pharmaceuticals (Underwood et al. 2015b).

By the end of the study, there was an overall increase in the liposome level present in each successive "Distribution" OFG. The highest levels of liposomes occurred in the 18 hour post-infusion group. This finding indicates that there is a positive correlation between time and liposome level (Underwood et al. 2015b). The 18 hour group was already showing signs of significant lameness, which may be too late for pharmaceutical intervention. However, Underwood et al. (2015b) suggest the dual use of liposomes and a known inhibitor of laminitis development, such as cryotherapy (van Eps and Pollitt 2004) On its own, cryotherapy is not a practical laminitis solution, as it is both time consuming and needs to be repeated often and/or maintained for extended periods of time. However, in order to establish an effective liposome delivery time line, it may be possible to combine liposome administration with cryotherapy in future laboratory studies (Underwood et al. 2015b).

IMPLICATIONS

The aim of "Distribution" was to discern if liposomes accumulate in lamellar tissue during oligofructose-induced laminitis. If liposomes did, then future research could use them as carriers for laminitis pharmaceuticals, which may intervene in laminitis development.

The Underwood et al. (2015b) experiment produced results which not only showed that laminitis development incurs an accumulation of liposomes in the lamellar tissue, but their data further supports the use of liposomes as a delivery method for pharmaceutical intervention of laminitis. This support is due to the high levels of circulating liposomes in the study groups, especially the OFG at 18 hours (Underwood et al. 2015b).

Underwood et al. (2015b) calculated that a significant concentration of a drug may be attainable (specifically 332 ng/ tissue gram) twelve hours after administration. These calculations exceed those found in the previous Underwood et al. (2015a) experiment using **regional limb perfusion** to administer the MMP-inhibitor marmistat. Underwood et al. (2015b)'s calculations indicate that there is a potential to inhibit 90% of the MMP-2 and MMP-9 in the lamellar tissue (Underwood et al. 2015ab).

Regional limb perfusion: Injecting a drug into the tissue, joint, or bone for increased concentration or specificity of application

Underwood et al. (2015b) go on to discuss other studies that produced results indicating a potential for a continued increase in liposome level, past the 12 hour cut-off in the "Distribution" study; along with the similarities and differences between their results and the results from other infection-caused inflammation studies. However, these other inflammation studies have a great many other variables to consider, to the point that they are not directly comparable to the "Distribution" study.

Finally, Underwood et al. (2015b) state that the results of "Distribution" may provide basis for further studies on liposome-delivered drugs in other species, due to the levels of RLL accumulated in the samples taken. The high RLL level viewed in the lamellar tissue, versus the other tissues sampled, indicate that the vascular permeability of these tissues are different. Most important for this primer is Underwood et al. (2015b)'s data that supports the potential for the first clinically applicable pharmaceutical intervention of laminitis.

CONCLUSION

Laminitis is a painful, debilitating disease that affects horses, horse owners, and veterinarians all over the world. The complex pathogenesis of laminitis involves many potential pathways, and the root of it is still being sought. Understanding how to treat the manifestations of laminitis will not only provide relief for suffering equines, but it can assist in finding what causes laminitis in the first place. The AELRU, along with their colleagues around the world, have been making great strides in their laminitis research. Linking sepsis, liposomes, MMP inhibitors, and laminitis is a remarkable start to reaching the end of this disease.

ACKNOWLEDGEMENTS

I would like to thank Dr. Judi Roux, Dr. Robert Sterner, and Dr. Elizabethada Wright for their support and contributions to this year's DJUB. I am especially grateful for their guidance as I navigated the role of being the editor. I thank my associate Murat Can Kalem for his assistance in getting this issue ready for publication. I wish to thank the peer reviewers who helped to improve this article. I thank John Rahkola for drafting my figures, and Alicia Boltz for making them publication-ready, along with all of her assistance in navigating InDesign. I thank JC for being my rock.

BIOGRAPHY

Tami Rahkola is a senior at the University of Minnesota Duluth. She is pursuing a B.S. in Biology, emphasis on organismal biology, and a minor in Environmental Science. After graduation, she would like to attend graduate school, and ultimately pursue a career related to natural resource or wildlife management. In her free time, Tami enjoys reading and writing, along with hiking, camping, fishing and horseback riding with her son.

REFERENCES

Arulsudar N, Subramanian N, Mishra P, Chuttani K, Sharma RK, Murthy RSR. 2004. Preparation, characterization, and biodistribution study of technetium-99m-labeled leuprolide acetate-loaded liposomes in Ehrlich ascites tumor-bearing mice. AAPS Pharm. 6(1):5.

Australian Equine Laminitis Research Unit [Internet]. 2015. University of Queensland (Aus): School of Veterinary science; [cited 2016 Mar 24]. Available from https://veterinary-science.uq.edu.au/australian-equine-laminitis-research-unit

Cawcutt KA, Peters SG. 2014. Severe sepsis and septic shock: Clinical overview and update on management. Mayo Clinic Proceed. 89(11):1572-1578.

Engiles JB, Galantino-Homer HL, Boston R, McDonald D, Dishowitz M, Hankenson KD. 2015. Osteopathology in the Equine distal phalanx associated with the development and progression of laminitis. Vet Pathol. 52(5):928-944.

Gardner AK, Kelly CS, van Eps AW, Burns TA, Watts MR, Belknap JK. 2015. Mitogen-activated kinase pathway activation in epidermal lamellae in the acute stages of carbohydrate overload laminitis models and the effect of regional deep hypothermia on signaling pathways. Equine Vet J. 0:1-8.

Holl HM, Gao S, Fei Z, Andrews C, Brooks SA. 2015. Generation of a de novo transcriptome from equine lamellar tissue. BioMed Central. 16:739-748.

Jiang R, Gao L, Wang G, Li X, Li Y, Fan X, Liu X, Wang J, Zhang Y, Kong X, Xiao J. 2015. Role of insulin during the development of oligofructose (OF)-induced equine laminitis. Bull Vet Inst in Pulawy. 59(2):303-309.

Isaka Y, Furukawa S, Etani H, Nakanishi E, Ooe Y, Imaizumi M. 2000. Noninvasive measurement of cerebral blood flow with 99mTc-Hexamethylpropyleneamine oxime single-photon emission computed tomography and 1-point venous blood sampling. Stroke. 31:2203-2207.

Kaczmarek K, Janicki B, Glowska M. 2016. Insulin resistance in the horse: a review. J App Animal Res. 44(1): 424-430.

Katz LM, Bailey SR. 2012. A review of recent advances and current hypothesis on the pathogenesis of acute laminitis. Equine Vet J. 44(6): 752-761.

Lameness and laminitis in US Horses. (United States Department of Agriculture). 2000. National Animal Health Monitoring System. Fort Collins (CO). #N318.0400.

Laskoski LM, Valdao CAA, Dittrich RL, Deconto I, Faleiros RR. 2016. An update on equine laminitis. Cienc. Rural. 46(3):547-553

Laverman P, Brouwers AH, Dams ETM, Oyen WJG, Storm G, Rooijen NC, Corstens FHM, Boerman OC. 2000. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. J Pharm Exp Therap. 293(3):996-1001.

Li X, Jiang R, Wang G, Li Y, Fan X, Liu X, Wang J, Pan J, Gao L. 2015. MMP-2 Plays an important role during the early acute developmental phase of Oligofructose-induced Equine laminitis. Bull Vet Inst in Pulawy. 59(1):149-153.

Medina-Torres CE, Underwood C, Pollitt CC, Castro-Olivera EM, Hodson MP, Richardson DW, van Eps AW. 2014. The effect of weightbearing and limb load cycling on equine lamellar perfusion and energy metabolism measured using tissue microdialysis. Equine Vet J. 48(2016):114-119.

Medina-Torres CE, Underwood C, Pollitt CC, Castro-Olivera EM, Hodson MP, Richardson DW, van Eps AW. 2015. Microdialysis measurements of lamellar perfusion and energy metabolism during the development of laminitis in the oligofructose model. Equine Vet J. 48(2016):114-119.

Oyen WJG, Boerman OC, Storm G, van Bloois L, Koenders EB, Claessens RAMJ, Perenboom RM, Crommelin DJA, van der Meer JWM, Corstens FHM. 1996. Detecting infection and inflammaiotn with technetium-99m-labeled stealthÆ Liposomes. J Nucl Med. 37(8): 1392-1397.

Pollitt CC. 2004. Anatomy and Physiology of the inner hoof wall. Clin Tech Equine Pract. 3(1):3-21.

Pollitt CC. 2010. The anatomy and physiology of the suspensory apparatus of the distal phalanx. Vet Clin Equine 26:29-49.

Szebeni J, Alving CR, Rosivall L, BÜnger R, Baranyi L, BedÖcs P, TÓth M, Barenholz Y. 2007. Animal models of complement-mediated hypersensitivity reactions to liposomes and other lipid-based nanoparticles. J Lipo Res. 17:107-117.

Tadros EM, Frank N, Newkirk KM, Donnell RL, Horohov DW. 2012. Effects of a "two-hit" model of organ damage on the systemic inflammatory response and development of laminitis in horses. Vet Immuno and Immunopath. 150(1-2):90-100.

Tadros EM, Frank N, Horohov DW. 2013. Inflammatory cytokine gene expression in blood during the development of oligofructose-induced laminitis in horses. J Equine Vet Sci. 33(10): 802-808

Underwood C, van Eps AW, Ross MW, Laverman P, van Bloois L, Storm G, Schaer TP. 2012. Intravenous technetium-99m labelled PEG-liposomes in horses: A safety and biodistribution study. Equine Vet J. 44:196-202.

Underwood C, van Eps AW. 2012. Nanomedicine and veterinary science: The reality and the practicality. Vet J. 193(1):12-23.

Underwood C, Collins SN, Mills PC, van Eps AW, Alla Vena RE, Medina-Torres CE, Pollitt CC. 2015. Regional intravenous limb perfusion compared to systematic intravenous administration for marimastat delivery to equine lamellar tissue. J of Vet Pharma and Therap. 38:392-399.

Underwood C, Pollitt CC, Metselaar JM, Laverman P, van Bloois L, van den Hoven JM, Storm G, van Eps AW. 2015. Distribution of technetium-99m PEG-liposomes during oligofructose-induced laminitis development in horses. The Vet J. 206(2015): 218-225.

Van Eps AW, Pollitt CC. 2004. Equine laminitis: cryotherapy reduces the severity of the acute lesion. Equine Vet J. 36(3):255-260.

Van Eps AW, Pollitt CC. 2006. Equine laminitis induced with oligofructose. Equine Vet J. 38(3):203-208.

Visser MN, Pollitt CC. 2012. The timeline of metalloprotease events during oligofructose induced equine laminitis development. Equine Vet J. 44:88-93.

Wattle O, Pollitt CC. 2004. Lamellar metabolism. Clin Tech Equine Pract. 3(1): 22-33.

Ziessman HA, O'Malley JP, Thrall JH. 2013. Radiopharmaceuticals. In: Elsevier Health Sciences. Nuclear medicine: The requisites. Philadelphia (PA): Elsevier. p. 1-15.

i *Please note: unless otherwise cited, all data and information between this disclaimer and the Conclusion comes from "Distribution of technetium-99m PEG-liposomes during oligofructose induced laminitis development in horses," by Underwood et al. (2015b).