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Distribution of technetium-99m PEG-liposomes during oligofructose-induced laminitis development in horses

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Highlights

- This study investigated whether polyethylene-glycol (PEG) coated liposomes accumulate in the lamellar tissue during laminitis development
- ^{99m}Tc-PEG-liposomes were administered to normal horses and horses with oligofructose-induced laminitis
- Scintigraphic examination and lamellar tissue collection demonstrated liposome accumulation in the lamellar tissue of horses with oligofructose-induced laminitis.
- Liposomes may have potential for targeted lamellar drug delivery in acute laminitis

Abstract

Liposomes are phospholipid nanoparticles used for targeted drug delivery. This study aimed to determine whether intravenous liposomes accumulate in lamellar tissue during laminitis development in horses so as to assess their potential for targeted lamellar drug delivery. Polyethylene-glycol (PEG) coated liposomes were prepared according to the film hydration method and labelled using ^{99m}Tc-hexamethyl-propylene-amine-oxime. Six horses received 10 g/kg oligofructose via nasogastric tube to induce laminitis, and four control horses received water via nasogastric tube. All horses received 300 µmol ^{99m}Tc-PEG-liposomes (5.5 GBq) plus 5.5 µmol/kg PEG-liposomes by slow intravenous infusion. Scintigraphic imaging was performed at 0, 6 and 12 h post-infusion. Technetium-99m liposome uptake was measured in regions of interest over the hoof, fetlock and metacarpus. At the study end-point horses were euthanased, tissue samples collected and tissue liposome levels were calculated as the percentage of the injected dose of ^{99m}Tc-liposomes per kilogram of tissue. Data were analysed non-parametrically.

All horses receiving oligofructose developed clinical and histological signs of laminitis. Technetium-99m liposome uptake in the hoof increased with time in laminitis horses (P=0.04), but decreased with time in control horses (P=0.01). Technetium-99m liposome levels in lamellar tissue from laminitis horses was 3.2-fold higher than controls (P=0.02) and were also higher in laminitis vs. control skin, muscle, jejunum, colon, and kidney (P<0.05). Liposomes accumulated in lamellar tissue during oligofructose-induced laminitis development and demonstrated potential for targeted lamellar drug delivery in acute laminitis. This study provides further evidence that lamellar inflammation occurs during laminitis development. Liposome accumulation also occurred in the skin, muscle, jejunum, colon and kidneys, suggesting systemic inflammation in this model.

Keywords: Nanoparticle, Horse, Liposome, Scintigraphy, Nanotechnology

Introduction

Acute laminitis is a crippling disease of the horse foot with irreversible pathology so prophylaxis is paramount. There are three broad aetiological categories of laminitis, namely, endocrinopathic, sepsis-related and supporting limb laminitis. Similar to sepsis-related organ failure in humans, sepsis-related laminitis often results in the demise of the patient after successful treatment of the primary condition. Sepsis is defined as documented or suspected infection, plus systemic manifestations of infection (Cawcutt and Peters, 2014).

The oligofructose model of laminitis involves administration of 10 g/kg oligofructose by nasogastric tube, resulting in clinical signs of enterocolitis, presumptive disruption of the gastrointestinal barrier, documented endotoxaemia and subsequent laminitis development (van Eps et al., 2006; Bailey et al., 2009). A systemic inflammatory response occurs mirroring that which occurs during human sepsis, including the classical symptoms of pyrexia, tachycardia, tachypnoea, haemoconcentration and leucocytosis (van Eps et al., 2006; Belknap, 2007; Bailey et al., 2009; Cawcutt and Peters, 2014). During the development of oligofructose-induced laminitis, endothelial activation, leukocyte infiltration and an increase in pro-inflammatory cytokine and cyclooxygenase (COX)-2 expression occur in the lamellae in common with findings seen in organ failure associated with human sepsis (Black et al., 2006; Belknap et al., 2007; Tadros et al., 2012). Therefore the oligofructose model is considered to be an appropriate model for naturally-occurring sepsis-related laminitis, such that occurs secondary to diseases such as colitis (Belknap et al., 2007; Belknap and Black, 2012; van Eps, 2012; Kullmann et al., 2014).

Distal limb cryotherapy prevents sepsis-related laminitis (Van Eps and Pollitt, 2004; van Eps et al., 2014; Kullmann et al., 2014), but it is labour-intensive and practically challenging. Potential therapeutic targets involved in the pathophysiology of sepsis-related laminitis have been identified and include inflammation (Belknap et al., 2007; Leise et al., 2011, 2012; Visser and Pollitt, 2011; Tadros et al., 2012), proteolytic enzyme activation (Pollitt et al., 1998; Visser and Pollitt, 2012; Wang et al., 2012) and wingless-related integration site (Wnt)-pathway dysregulation (Wang et al., 2013). Pharmaceuticals directed to address these targets are available and include non-steroidal anti-inflammatory drugs, corticosteroids, signal transducer and activator of transcription (STAT) 3 inhibitors (Leise et al., 2012), protease inhibitors (Pollitt et al., 1998; De Savi et al., 2011) and Wnt-pathway agonists (Wang et al., 2013). Evaluation of these substances for laminitis prophylaxis has been hindered by their unsuitability for systemic delivery due to cost, rapid clearance, low bioavailability, inability to achieve therapeutic tissue concentrations, unwanted systemic side effects and/or degradation in the circulation (Levin et al., 2006; Nourian et al., 2010).

Recent work has investigated methods of regional lamellar delivery (Nourian et al., 2010; Underwood et al., 2015; C. Underwood et al, unpublished data). Despite initial promising results (Nourian et al., 2010), intraosseous infusion of the distal phalanx does not consistently yield therapeutic lamellar drug concentrations (C. Underwood et al., unpublished data). Regional limb perfusion requires frequent dosing (of each limb separately) to maintain therapeutic concentrations (Underwood et al., 2015), so the technique is not suitable for widespread clinical application. A means of lamellar drug delivery that provides sustained, therapeutic lamellar drug concentrations and has potential for clinical application is required.

Nanoparticles are ordered structures used for targeted drug delivery. They improve the therapeutic index and safety profile of the substances they carry (Cordeiro et al., 2000; Metselaar et al., 2003; Hofheinz et al., 2005; Rose et al., 2005). Nanoparticles also provide a means of sustained delivery over a period of days or even weeks (Sahoo and Labhasetwar, 2003; Bakker-Woudenberg et al., 2005; Fahmy et al., 2005). As a consequence, nanoparticle formulations may require a reduced dose compared to free drug, thus reducing the cost of expensive pharmaceuticals (Underwood and van Eps, 2012).

Liposomes are phospholipid nanoparticles that form highly flexible delivery systems with the ability to carry both hydrophilic and hydrophobic pharmaceuticals. Their safety and biodistribution has been established in normal horses (Underwood et al., 2012). Small polyethylene-glycol (PEG) coated liposomes are most suitable for targeted drug delivery after intravenous (IV) administration. Following injection, a relatively small number of PEG-liposomes are endocytosed by macrophages. Those evading endocytosis remain in circulation for prolonged periods of time (Arulsudar et al., 2004). Together with their small size, these long-circulating characteristics confer the ability to extravasate at sites of increased vascular permeability and facilitate accumulation in the tissue around those sites (Laverman et al., 2001). This has been termed the enhanced permeability and retention (EPR) effect and is the underlying principle behind many passively targeted nanoparticle drug delivery systems.

Increased vascular permeability occurs at sites of inflammation that occurs during laminitis development (Belknap et al., 2007; Visser, 2009; Faleiros et al., 2011; Tadros et al., 2012). Therefore, liposomes may accumulate in lamellar tissue during laminitis development and provide a means of targeted lamellar drug delivery. The objectives of this study were (1)

to establish whether liposomes accumulate in the lamellar tissue during laminitis development in the oligofructose (OF) model of laminitis (a sepsis model), and (2) to establish the biodistribution of liposomes during sepsis in the OF model.

Materials and methods

The project was approved by the University of Queensland Animal Ethics Committee (approval number: SVS/117/11) that monitors compliance with the Animal Welfare Act (2001) and The Code of Practice for the care and use of animals for scientific purposes (current edition).

Animals and laminitis induction

Ten mature Standardbred geldings (aged 4-11 years, 395-583 kg bodyweight), with no lameness and no gross nor radiographic abnormalities of the feet were enrolled in the study. The horses were housed and fed in stables for 4 weeks prior to the experiment as previously described (van Eps and Pollitt, 2006).

During the experiment the horses were housed in climate-controlled, lead lined stalls and had ad libitum access to hay and water. Six horses (laminitis group) received alimentary overload with OF (bolus dose of 10 g/kg OF via nasogastric tube, up to a maximum dose of 4.2 kg) to induce laminitis as previously described (van Eps and Pollitt, 2006). Four control horses (control group) received equivalent volumes of plain water by nasogastric tube. The horses were monitored every 2 h throughout the study and heart rates, respiratory rates and temperatures were recorded every 6 h. Upon onset of Obel grade 2 lameness, defined as

lameness detectable at the walk (Obel, 1948), a single dose of phenylbutazone (Phenylarthrite, Ausrichter) was administered IV at 8 mg/kg.

Liposome preparation

Dipalmitoyl phosphatidylcholine (DPPC) and PEG-(2000)-distearoyl phosphatidylethanolamine (PEG-(2000)-DSPE) were obtained from Lipoid, cholesterol and glutathione (GS-H) were obtained from Sigma. All chemicals were of reagent grade. A chloroform/methanol mixture (10:1 volumetric ratio) containing DPPC, PEG-(2000)-DSPE and cholesterol was prepared at a molar ratio of 1.85:0.15:1. A lipid film was formed by rotary evaporation followed by nitrogen flushing to remove residual organic solvent. The lipid film was dispersed at 50 °C in 100 mmol/L GS-H in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (10 mmol/L HEPES, 135 mmol/L NaCl, pH 7.5) at a total lipid concentration of 100 mmol/L. Empty liposomes were dispersed in HEPES buffer only.

The liposomes were sequentially sized by extrusion using a high pressure extruder (Lipex) with polycarbonate filters of 200, 100, 80 and 50 nm pore size (Whatman International). Unentrapped GS-H was removed by dialysis against HEPES buffer using Slide-A-Lyzer dialysis cassettes with a molecular cut-off of 10 kDa (Pierce). Finally, the liposomes were passed through a 0.2 mm sterile disc filter. The mean particle size was determined by dynamic light-scattering with an ALV CGS-3 system (Malvern instruments). The liposomes had a mean size of 85 nm with a polydispersity index of 0.139. Phospholipid content was determined with a phosphate assay (Fiske, 1925; Rouser et al., 1970). The final liposome preparations contained an average of 33.3 mg total lipid/mL.

176

177 *Radiolabelling of liposomes*

178 Preformed G-SH-PEG-liposomes were labelled by transporting ^{99m}Tc as a lipophilic
179 ^{99m}Tc-hexamethylpropylene-amine-oxime (HMPAO) complex through the lipid bilayer as
180 described previously (Underwood et al., 2012). The ^{99m}Tc-HMPAO was irreversibly trapped
181 in the internal aqueous phase due to reduction by the encapsulated G-SH (Oyen et al., 1996).
182 For each dose, 8.5 GBq of ^{99m}Tc was incubated with 1 mg HMPAO (Ceretec, GE
183 Healthcare). A dose calibrator (Atom Lab 400, Biodex) was used to measure the activity of
184 liposomal and non-bound ^{99m}Tc-HMPAO to enable calculation of labelling efficiency.

185

186 *Liposome administration and monitoring procedures.*

187 All horses received 300 µmol (phospholipid) ^{99m}Tc-PEG-liposomes and 5.5 µmol
188 phospholipid/kg bodyweight unlabelled PEG-liposomes in 1 L 0.9% NaCl via IV catheter
189 into the left jugular vein. Unlabelled liposomes were administered to increase the total
190 phospholipid dose to a level above that at which rapid clearance is seen (Laverman et al.,
191 2000). In the laminitis group two horses received liposomes immediately after OF
192 administration (LAM-0), two received liposomes 12 h after OF administration (LAM-12) and
193 two received liposomes 18 h after liposome administration (LAM-18). The liposomes were
194 infused slowly to avoid complement mediated hypersensitivity reactions (Szebeni, 2005).
195 Heart rate, respiratory rate and rectal temperature were monitored every 10 min for 60 min
196 from the start of the infusion.

197

Imaging studies

Scintigraphic examinations were performed in all horses at 1, 6 and 12 h after the start of the liposome infusion (p.i.) using a large field-of-view scintillation camera with a high resolution collimator (Technicare Omega 500 gamma camera, GE Healthcare) in a climate controlled lead-lined room. Scans were also performed at 18 and 24 h p.i. in the LAM-0 group. Post-acquisition nuclear medicine software was used for image display and analysis (NuQuest v3.0 imaging software, MedX).

Three-minute static images of the dorsal and lateral aspects of both forefeet were acquired at each time point with the hooves positioned a set distance from the camera. Images were stored in a 256 × 256 matrix and were analysed retrospectively by a blinded observer (CU) with all measurements performed in triplicate.

The count density was established in regions of interest (ROI) over the hoof, fetlock, and metacarpus in each image. The count density per second (CD/s) was calculated, and corrected for both the decay and the total initial dose in each horse. The ratio of the count densities in the hoof and metacarpus (hoof:metacarpus) and the fetlock and metacarpus (fetlock:metacarpus) in each horse were calculated. Hoof wall surface temperature (HWST) was recorded before each examination using a hand-held infra-red scanner (Exergen).

Liposome biodistribution

Blood samples were collected directly from the right jugular vein immediately following liposome infusion and at 6 and 12 h p.i. Blood radioactivity was measured on a

shielded well scintillation gamma counter (Ludlum Instruments) and corrected for volume and decay. The half-life of ^{99m}Tc-liposomes was roughly estimated by non-compartmental analysis using a log-linear model on PKSolver (China Pharmaceutical University).

At the end of the study (immediately following the 12 h scintigraphic examination in the LAM-12 and LAM-18 groups and the 24 h scintigraphic examination in the LAM-0 group) the horses were examined for lameness at the walk. Lameness was scored according to the Obel grading system (Obel, 1948). The horses were euthanased with pentobarbital sodium (20 mg/kg IV). Samples of lung, liver, spleen, kidney, jejunum, colon, forelimb lamellar tissue, muscle, skin and adipose tissue were dissected immediately following euthanasia. Samples were weighed and the activity of the sample (equivalent to the amount of ^{99m}Tc-liposomes present in the sample (Phillips et al., 1992) was measured using a shielded well scintillation gamma counter (Ludlum instruments). To correct for physical decay, an aliquot of the original injected dose was counted simultaneously. The results were expressed as percentage injected dose/kg of tissue (% ID/kg).

Histological evaluation

Dorsal lamellar sections from the forelimbs were fixed using 10% neutral buffered formalin, processed by routine paraffin embedding, sectioned at 4 µm and stained with haematoxylin and eosin and periodic acid–Schiff for light microscopy as previously described (Pollitt, 1996). Histological analysis was performed by a blinded observer (AVE). The severity of laminitis pathology was scored for each section using a system based on the 0-3 scale previously described by Pollitt (Pollitt, 1996).

Data analyses

Data were analysed using a statistical software package (GraphPad Prism 6). The data were tested for normality using D'Agostino-Pearson omnibus normality tests. Data distributions were either non-Gaussian, or group numbers were too small to presume a normal distribution, hence non-parametric tests were used. Non-paired data were compared by Mann *U* Whitney tests. Comparisons of repeated measured were analysed using Friedman analyses with Dunn's post-tests. For the analysis of scintigraphic data only data from the final 12 h of the LAM-0 group were used. Significance was set at $P \leq 0.05$. Unless otherwise stated, data are expressed as median [interquartile range].

Results

All horses that received OF (the laminitis group) developed fever, diarrhoea and lameness (Obel grade 2-3). Median histological scores were significantly greater in the laminitis group (1.5[1-2]) compared to the control group (0[0-0.38], P=0.01).

Radiolabelling efficiency was 82[63-90]% and the median activity of the ^{99m}Tc-liposomes immediately prior to infusion was 5.5[4.5-6.6] GBq. There was no difference in the median radioactivity of the ^{99m}Tc-liposomes administered to laminitis versus control horses (5.0[4.2-6.8] GBq vs. 6.1[4.8-6.4] GBq, respectively). Each horse received the full liposome dose and the median infusion time was 38[35-49] min. No adverse reactions were detected and there were no significant changes in heart rate, respiratory rate or temperature during the infusion (data not shown).

Based on the radioactivity in sequential blood samples (corrected for decay), the ^{99m}Tc-liposomes exhibited slow elimination from the circulation with a median half-life of 22.9[8.4-32.4] h. Due to the limited number of time-points, these half-lives are only a rough estimate. There was no difference in the median half-life between the laminitis and control groups. There was a decrease in the blood radioactivity (corrected for decay) with time in the laminitis group (P<0.001; Fig. 1). At 12 h p.i. the blood radioactivities in laminitis horses were lower than those in control horses (P=0.01; Fig. 1).

The scintigraphic images demonstrated subjective diffuse increased ^{99m}Tc-liposome uptake in the hoof and distal limb of laminitis horses compared to controls; evident at 6 h, and more marked at 12 h p.i. (Fig. 2). In the laminitis group the CD/s increased with time in the dorsal hoof and fetlock ROIs (P<0.01; Figs. 3A and B). Conversely in control horses the CD/s in the dorsal hoof, fetlock and metacarpal ROIs decreased with time, and the CD/s in the lateral hoof ROI also decreased with time p.i (P<0.05; Figs. 3 A-C). The CD/s in the lateral hoof ROIs were higher in laminitis horses compared to controls at 6 and 12 hours p.i. (P=0.02; Fig. 3D). The lateral hoof:metacarpal and fetlock:metacarpal CD/s ratios were higher in laminitis horses compared to controls at 12 h (1.9[1.8-2.0] vs. 1.5[1.4-1.6] and 1.5[1.4-1.8] vs. 1.1[1.0-1.1], respectively, P=0.03). There was no change in HWST with time, and there was no difference in HWST between laminitis and control horses at any time-point.

The ^{99m}Tc-liposomal levels in lamellar tissue of laminitis horses were higher than those in controls (0.21[0.14-0.3] %ID/kg vs. 0.065[0.06-0.11] %ID/kg, respectively; P=0.019; Fig. 4). Lamellar ^{99m}Tc-liposome levels were highest when the liposomes were administered at 18 h p.i; with a 4.8 fold increase compared to control horses (Fig. 5), however the numbers were too small for statistical analyses. There were increased ^{99m}Tc-liposome levels in the skin, muscle, jejunum, colon and kidney of laminitis horses compared to controls (P<0.05; Fig. 4). There was a trend towards increased ^{99m}Tc-liposome levels in the liver of laminitis horses compared to controls, and towards decreased ^{99m}Tc-liposome levels in the blood of laminitis horses compared to controls (P=0.06; Fig. 4).

Discussion

This study demonstrates that liposomes accumulate in the lamellar tissue during the development of sepsis-related laminitis using the OF experimental model. This conclusion is supported by both the scintigraphic imaging studies and the tissue biodistribution data. Previous studies have reported evidence of lamellar inflammation during laminitis development (Black et al., 2006; Belknap et al., 2007; Leise et al., 2011; Visser and Pollitt, 2011; Tadros et al., 2012). The accumulation of liposomes in lamellar tissue provides further evidence that lamellar inflammation and increased vascular permeability occurs during laminitis development in the OF model.

The primary objective of this study was to evaluate whether liposomes accumulate in (and have potential for) targeted lamellar drug delivery. The increased lamellar liposome levels in laminitis horses at 12 h p.i indicates that liposomal drug delivery systems have

potential to yield sustained lamellar drug concentrations. Recent studies performed in our laboratory investigated lamellar marimastat concentrations after IV administration of 0.23 mg/kg bodyweight (104 mg/450 kg horse) (Underwood et al., 2015). If the same dose was administered in liposomes at 18 h post-OF, based on the lamellar liposome levels in the LAM18 group (0.31% ID/kg), the lamellar marimastat concentration would be 332 ng/g tissue at 12 h post injection.

Although these calculations are merely speculative, this exceeds both the concentrations achieved 12 h after RLP and the concentration necessary for inhibition of 90% of lamellar MMP-2 and MMP-9 (Underwood et al., 2015; C. Underwood et al., unpublished data). Additionally, as reported in previous studies investigating liposome accumulation in inflammation, liposomes would probably continue to accumulate in the following 12h resulting in further increases in lamellar drug concentrations (Oyen et al., 1996; Boerman et al., 1997; Erdogan et al., 2000).

There were no adverse reactions to liposome administration, and no changes in heart rate, respiratory rate or temperature; adding to the evidence from previous studies (Underwood et al., 2012; Burton, 2013) that slow IV liposome administration is safe in horses. Therefore, based on this preliminary study liposomes appear to have potential for lamellar drug delivery.

The subjective increase in lamellar liposome levels between LAM-0, LAM-12 and LAM-18 groups, respectively, suggests there is a temporal increase in lamellar inflammation

post-OF administration, consistent with previous reports (Visser, 2009). Liposomes were administered immediately following, then at 12 h and 18 h after OF administration to achieve high circulating liposome levels coinciding with the onset of lamellar inflammation (Visser, 2009). The highest uptake was achieved when liposomes were administered at 18 h post-OF. By 18 h the horses were exhibiting Obel grade 1-2 lameness, which may be too late for delivery of laminitis prophylactics. However, prophylactic digital cryotherapy is effective when initiated at Obel grade 2 lameness (18.5 h post-OF administration; van Eps et al., 2014), so it is possible that a pharmaceutical means of laminitis prophylaxis would still be effective at this stage. Further work is needed to evaluate the optimal time-point for administration of liposomal-formulations of drugs for laminitis prophylaxis.

The 3.2 fold increase in the liposome levels in the lamellar tissue of laminitis horses compared to controls is somewhat lower than that the 24-fold increase reported in infected muscle in an equine focal infection model (Underwood, 2011). This initially seems somewhat discouraging; however, there are three key points to consider: firstly, liposome accumulation depends on the intensity of the inflammatory response (Oyen et al., 1996); focal infection models involve established inflammation, whereas in the present study liposomes were administered before or during the early stages of inflammation. Secondly, the biodistribution data in the focal infection study was obtained at 24 h p.i. whereas most of the data in this study were collected at 12 h p.i. (LAM-12 and LAM-18). Liposomes accumulate gradually with time (Boerman et al., 1997; Oyen et al., 1996). In a mouse focal infection model the abscess:control muscle ratio increased from 2.2 at 6 h p.i., to 11.7 at 24 h p.i. (Erdogan et al., 2000); therefore, it is probable if sampled at a later time-point there would be greater lamellar accumulation of liposomes. Thirdly, as lamellar inflammation and laminitis pathology are focused at the dermoepidermal interface (Pollitt, 1996; Faleiros et al., 2011), greater liposome

accumulation may be present at this target site than in the lamellar tissue as a whole (Metselaar et al., 2003).

Acute laminitis shares many characteristics with sepsis-related organ failure and systemic inflammatory response syndrome (SIRS) in humans (Belknap and Black, 2012; Tadros et al., 2012; Cawcutt and Peters, 2014). The accumulation in skin, muscle, jejunum colon and kidney, plus the trend towards increased hepatic liposome levels is supportive of a systemic inflammatory response resulting in inflammation and increased vascular permeability in these organs. To the authors' knowledge this is the first study to investigate liposomal biodistribution in an animal sepsis model.

The accumulation of liposomes in multiple organs in our study indicates that liposomes may have potential for targeted drug delivery to prevent sepsis related organ failure in other species. It is, however, interesting that there was a more profound increase in liposome accumulation (compared with controls) in the lamellar tissue compared with all the other studied tissues. This indicates that the inflammatory response and increase in vascular permeability in lamellar tissue are unique.

Increased interleukin (IL)-1β, IL-6 IL-8, IL-10 and tumour necrosis factor (TNF)-α gene expression has been reported previously in liver and lung of horses with OF-induced laminitis (Tadros et al., 2012). Interestingly, in the present study, there was no evidence of liposome accumulation in the lung. Normal horses demonstrate high pulmonary liposome uptake (Underwood et al., 2012), most probably due to endocytosis of liposomes by the

383 increased numbers of pulmonary intravascular macrophages (PIMs) present in the horse
384 (Longworth et al., 1994). During sepsis, PIMs are activated and endocytose
385 lipopolysaccharide and bacteria (Tsokos, 2003; Parbhakar et al., 2005). Therefore, it is
386 probable that during the development of OF-induced laminitis PIMs have already been
387 activated and are unavailable to endocytose liposomes. This reduction in liposomal
388 endocytosis may counter-balance the liposomes extravasating into the pulmonary
389 parenchyma due to inflammation-associated increases in vascular permeability.

390

391 Interestingly, the median lung uptake in the LAM-18 group appeared lower than that
392 in the LAM-0 group (1.2[0.95-1.45] % ID/kg vs. 1.87 [1.59-2.15] % ID/kg, respectively),
393 potentially indicative of reduced liposomal endocytosis uptake at 18 h due to saturation of
394 PIMs in the systemic inflammatory response prior to liposome administration. However, the
395 numbers were too small for statistical analyses so further studies are necessary to evaluate
396 this hypothesis.

397

398 Limitations of our study include the small numbers of horses, particularly the low
399 numbers in each of the LAM groups, and the limited study duration of only 12 h p.i. Further
400 investigations of liposomal delivery systems for sustained lamellar drug delivery are
401 warranted. These should focus on determining free and liposome-encapsulated drug
402 concentrations at multiple time-points over a prolonged period (>24 h). When considering the
403 scintigraphic data, it is important to note that the radiopharmaceutical uptake in the hoof ROI
404 includes all sources of radioactivity within the hoof, including blood in the vascular system,
405 along with a mild shielding effect of the hoof wall. Changes in perfusion occur during
406 laminitis development and may have affected scintigraphic data (Van Eps and Pollitt, 2004).

Although, artefactual alterations in the scintigraphic data due to changes in perfusion cannot be definitively ruled out, the lack of significant changes in HWST (an indicator of digital vascular perfusion), and the reduction in blood activity with time suggest the increase in CD/s in the hoof ROIs of laminitis horses was attributable to liposome accumulation rather than increased perfusion. The tissue biodistribution data more accurately represent liposome levels in specific tissues. However, these could still have been altered by residual blood in the tissue and changes in lamellar perfusion. Perfusion of the disarticulated limbs with saline may have further helped to remove any residual blood from the lamellar tissue. This was attempted in one forelimb of the initial two horses, however, it was not practical and did not appear to alter the % ID/kg lamellar tissue (0.09 [0.08-0.1]% in the perfused limbs vs. 0.07 [0.07-0.08] % in the non-perfused limbs). Therefore, it was considered unlikely that changes in perfusion had a significant effect on lamellar biodistribution data; further investigation would be needed to definitively rule this out.

Conclusions

IV liposome administration to horses with OF-induced laminitis resulted in liposome accumulation in the lamellar tissue. The degree of liposome accumulation indicates that liposomes have potential for targeted drug delivery to lamellar tissue. With further experimental validation liposomal drug delivery systems could be the first clinically applicable method for delivering sustained therapeutic concentrations of pharmaceuticals with lamellar targets, including drugs with potential for laminitis prophylaxis, analgesics and anti-inflammatories. Liposomes also accumulate in other tissues undergoing inflammation and may therefore be of value as a drug delivery system in sepsis related organ failure in other species.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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591 **Figure legends**

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593 Fig. 1. Decay corrected radioactivity in sequential blood samples following ^{99m}Tc-liposome
594 infusion in laminitis (grey circles) and control (black squares) horses. At 12 h post injection
595 (p.i.) the blood radioactivity in the laminitis group was lower than that in the control group
596 (*; *P=0.01*), and lower than the activity immediately p.i. in the laminitis group (^; *P=0.001*).
597 Data are expressed as medians ± interquartile range.

598

599 Fig. 2. Dorsal scintigraphic images from one of the control horses (A-C) and one of the
600 laminitis horses (D-F) in the study. Images A and D were obtained at 1 h post injection (p.i.),
601 B and E at 6 h p.i. and C&F at 12 h p.i. Images are corrected for acquisition time, decay and
602 initial dose.

603

604 Fig. 3. Variations in the median count density/s (CD/s) (± inter-quartile range), in
605 scintigraphic regions of interest (ROIs), in laminitis (grey circles) and control (black squares)
606 horses over time. All data are corrected for decay and initial dose. There was an increase in
607 the CD/s in dorsal hoof (A) and dorsal fetlock (B) ROIs of laminitis horses over time (*;
608 $P<0.01$) and a decrease in the CD/s in the dorsal hoof (A) and fetlock (B) and metacarpal (C)
609 ROIs of control horses over time (*; $P<0.01$).). The CD/s in the lateral hoof ROI (D) of
610 control horses decreased with time, (*; $P<0.05$) and was lower than that in laminitis horses at
611 6 and 12 h post injection (^; $P=0.02$). Data are expressed as medians ± interquartile range.

612

Fig. 4. The median liposome levels (expressed as percentage injected dose per kilogram [% ID/kg]) in various tissues in laminitis vs. control horses (LAM-0 [triangles], LAM-12 [circles], LAM-18 [diamonds] and control groups [black squares]). The % ID/kg was higher in lamellae (A), skin (B), muscle (C), jejunum (D), colon (E) and kidney (F) of laminitis horses ($P<0.05$). There were trends towards an increased % ID/kg in the liver (G) and a decreased % ID/kg in the blood (H) of laminitis horses ($P=0.06$).

Fig. 5. Lamellar liposome levels appeared to increase when liposomes were administered at later time-points post-oligofructose administration (LAM-0: horses that received liposomes at the time of oligofructose administration; LAM-12: horses that received liposomes12 h after oligofructose administration; LAM-18: horses that received liposomes 18 h after OF administration; CON: control horses that did not receive oligofructose). The bars are labelled with the lamellar liposome levels in that group compared to controls. Data are expressed as medians ± interquartile range.