



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

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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-hexamethylpropylene-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 μ mol ^{99m}Tc-PEG-liposomes (5.5 GBq) plus 5.5 μ mol/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 were 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.

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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 and Pollitt, 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 and Pollitt, 2006; Belknap et al., 2007; Bailey et al., 2009; Cawcutt and Peters, 2014). During the development of oligofructose-induced laminitis, endothelial activation, leucocyte 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

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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; Kullmann et al., 2014; van Eps 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 have 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 de-

velopment 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). Untrapped 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 µm 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 and Subbarow, 1925; Rouser et al., 1970). The final liposome preparations contained an average of 33.3 mg total lipid/mL.

Radiolabelling of liposomes

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

Liposome administration and monitoring procedures

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

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 ratios 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 (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–Whitney *U* tests. Comparisons of repeated measures 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 grades 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 vs. 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

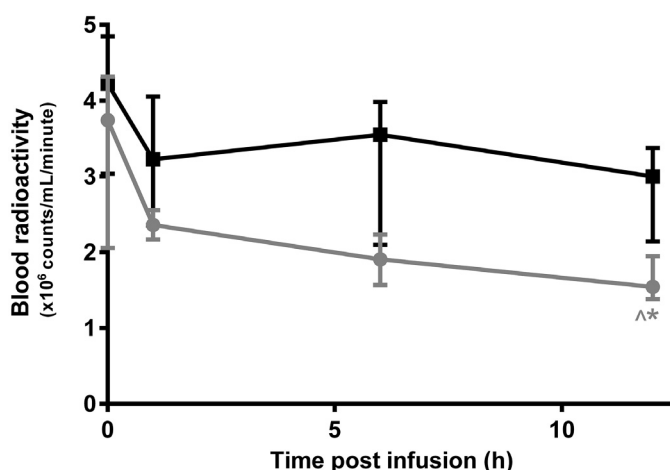


Fig. 1. Decay corrected radioactivity in sequential blood samples following ^{99m}Tc -liposome infusion in laminitis (grey circles) and control (black squares) horses. At 12 h post injection (p.i.) the blood radioactivity in the laminitis group was lower than that in the control group (*; $P = 0.01$),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

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, 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. 3A–C). The CD/s in the lateral hoof ROIs were higher in laminitis horses compared to controls at 6 and 12 h 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

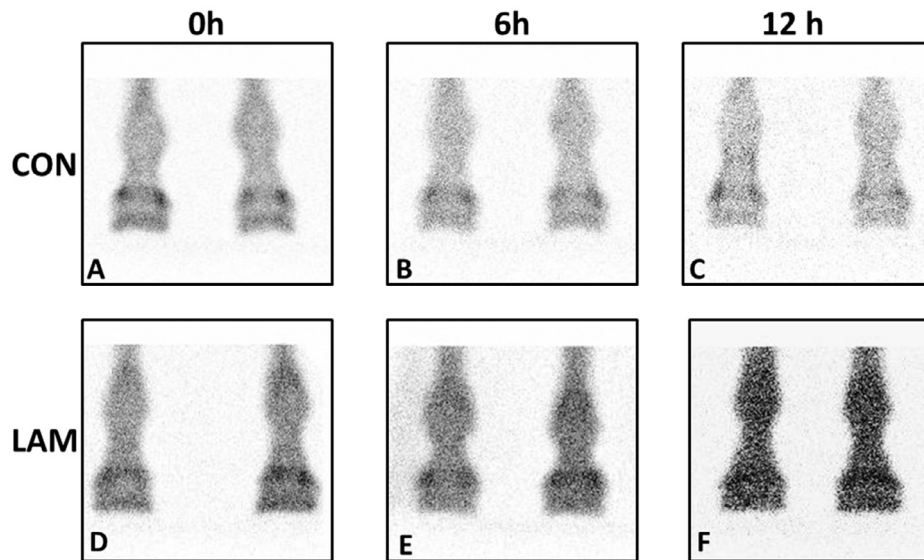


Fig. 2. Dorsal scintigraphic images from one of the control horses (A–C) and one of the laminitis horses (D–F) in the study. Images A and D were obtained at 1 h post injection (p.i.), B and E at 6 h p.i. and C and F at 12 h p.i. Images are corrected for acquisition time, decay and initial dose.

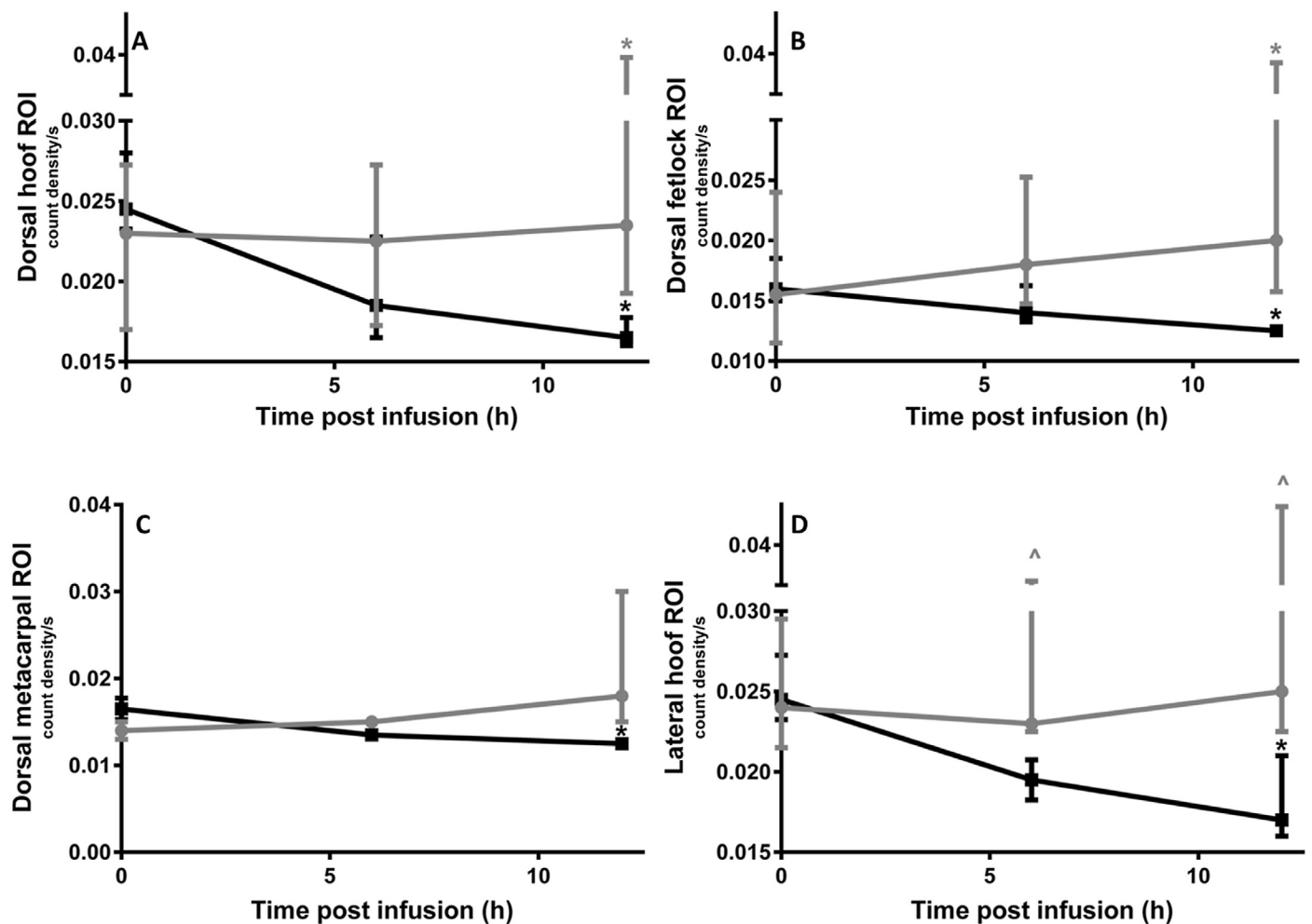


Fig. 3. Variations in the median count density/s (CD/s) (\pm inter-quartile range), in scintigraphic regions of interest (ROIs), in laminitis (grey circles) and control (black squares) horses over time. All data are corrected for decay and initial dose. There was an increase in the CD/s in dorsal hoof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

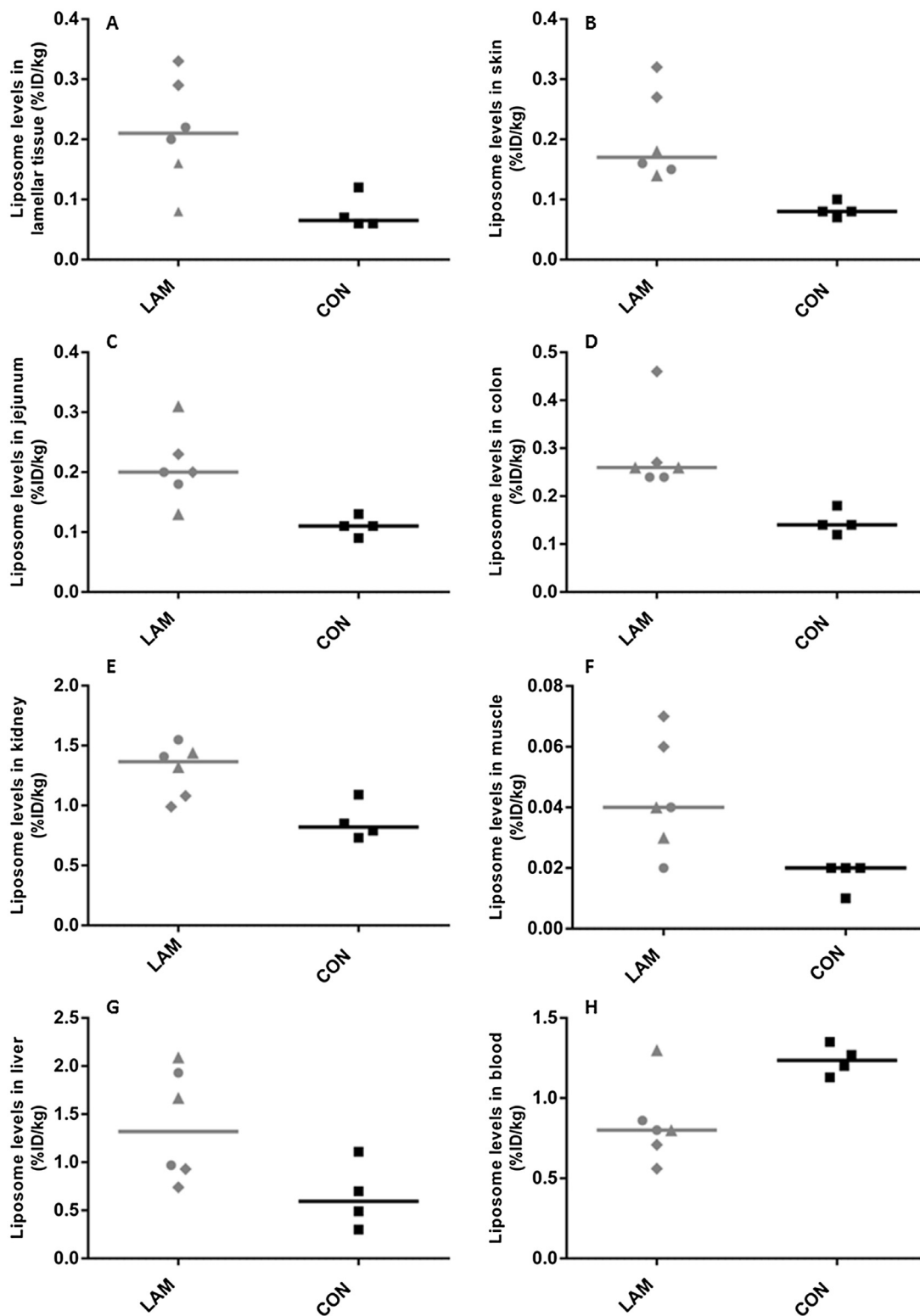


Fig. 4. The median liposome levels (expressed as percentage injected dose per kilogram [% ID/kg]) in various tissues in laminitis vs. control horses (LAM-0 [triangles], LAM-12 [circles], LAM-18 [diamonds] and control groups [black squares]). The % ID/kg was higher in lamellae (A), skin (B), muscle (C), 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 ($P = 0.06$).

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

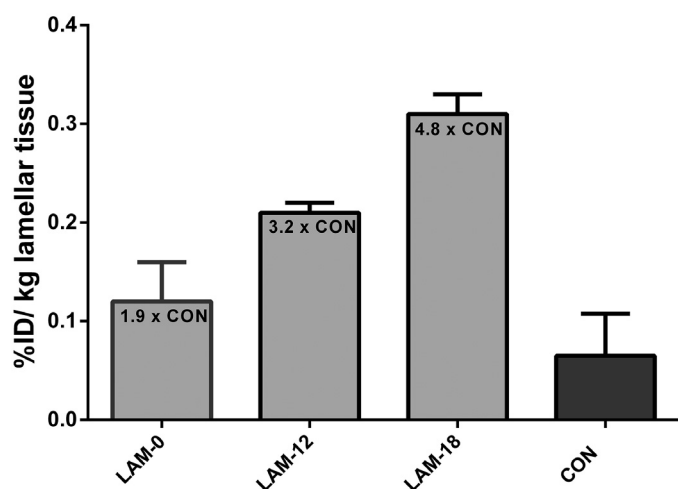


Fig. 5. Lamellar liposome levels appeared to increase when liposomes were administered at later time-points post-oligofructose administration (LAM-0: horses that received liposomes at the time of oligofructose administration; LAM-12: horses that received liposomes 12 h after oligofructose administration; 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 \pm interquartile range.

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 12 h 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 et al., 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 grades 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 ad-

ministration 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 et al., 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 were 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 (Oyen et al., 1996; Boerman et al., 1997). 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)-1 β , IL-6 IL-8, IL-10 and tumour necrosis factor (TNF)- α gene expression has been reported previously in the 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 increased numbers of pulmonary intravascular macrophages (PIMs) present in the horse (Longworth et al., 1994). During sepsis, PIMs are activated and endocytose lipopolysaccharide and bacteria (Tsokos, 2003; Parbhakar et al., 2005). Therefore, it is probable that during the development of OF-induced laminitis PIMs have already been activated and are unavailable to endocytose liposomes. This reduction in liposomal endocytosis may counter-balance the liposomes extravasating into the pulmonary parenchyma due to inflammation-associated increases in vascular permeability.

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

Limitations of our study include the small numbers of horses, particularly the low numbers in each of the LAM groups, and the limited study duration of only 12 h p.i. Further investigations of

liposomal delivery systems for sustained lamellar drug delivery are warranted. These should focus on determining free and liposome-encapsulated drug concentrations at multiple time-points over a prolonged period (>24 h). When considering the scintigraphic data, it is important to note that the radiopharmaceutical uptake in the hoof ROI includes all sources of radioactivity within the hoof, including blood in the vascular system, along with a mild shielding effect of the hoof wall. Changes in perfusion occur during 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 that 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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References

- Animal Welfare Act, 2001. <https://www.legislation.qld.gov.au/LEGISLTN/CURRENT/A/AnimalCaPrA01.pdf>.
- Arulsudar, N., Subramanian, N., Mishra, P., Chuttani, K., Sharma, R.K., Murthy, R.S., 2004. Preparation, characterization, and biodistribution study of technetium-99m-labeled leuprolide acetate-loaded liposomes in Ehrlich ascites tumor-bearing mice. *The AAPS Journal* 6, 45–56.
- Bailey, S.R., Adair, H.S., Reinemeyer, C.R., Morgan, S.J., Brooks, A.C., Longhofer, S.L., Elliott, J., 2009. Plasma concentration of endotoxin and platelet activation in the developmental stage of oligofructose-induced laminitis. *Veterinary Immunology and Immunopathology* 129, 167–173.
- Bakker-Woudenberg, I.A., Schiffelers, R.M., Storm, G., Becker, M.J., Guo, L., 2005. Long-circulating sterically stabilized liposomes in the treatment of infections. *Methods in Enzymology* 391, 228–260.
- Belknap, J.K., Black, S.J., 2012. Sepsis-related laminitis. *Equine Veterinary Journal* 44, 738–740.
- Belknap, J.K., Giguere, S., Pettigrew, A., Cochran, A.M., Van Eps, A.W., Pollitt, C.C., 2007. Lamellar pro-inflammatory cytokine expression patterns in laminitis at the developmental stage and at the onset of lameness: Innate vs. adaptive immune response. *Equine Veterinary Journal* 39, 42–47.
- Black, S.J., Lunn, D.P., Yin, C.L., Hwang, M., Lenz, S.D., Belknap, J.K., 2006. Leukocyte emigration in the early stages of laminitis. *Veterinary Immunology and Immunopathology* 109, 161–166.
- Boerman, O.C., Oyen, W.J., Storm, G., Corvo, M.L., van Bloois, L., van der Meer, J.W., Corstens, F.H., 1997. Technetium-99m labelled liposomes to image experimental arthritis. *Annals of the Rheumatic Diseases* 56, 369–373.
- Burton, A.J., Giguere, S., Sullivan, K., Arnold, R.D., 2013. Pharmacokinetics of intravenous and nebulized liposome-encapsulated gentamicin sulfate in foals. In: *Proceedings of the American College of Veterinary Internal Medicine*, Seattle, WA, USA, p. 653.
- Cawcutt, K.A., Peters, S.G., 2014. Severe sepsis and septic shock: Clinical overview and update on management. *Mayo Clinic Proceedings* 89, 1572–1578.
- Cordeiro, C., Wiseman, D.J., Lutwyche, P., Uh, M., Evans, J.C., Finlay, B.B., Webb, M.S., 2000. Antibacterial efficacy of gentamicin encapsulated in pH-sensitive liposomes against an in vivo *Salmonella enterica* serovar typhimurium intracellular infection model. *Antimicrobial Agents and Chemotherapy* 44, 533–539.
- De Savi, C., Pape, A., Cumming, J.G., Ting, A., Smith, P.D., Burrows, J.N., Mills, M., Davies, C., Lamont, S., Milne, D., et al., 2011. The design and synthesis of novel N-hydroxyformamide inhibitors of ADAM-TS4 for the treatment of osteoarthritis. *Bioorganic and Medicinal Chemistry Letters* 21, 1376–1381.
- Erdogan, S., Ozer, A.Y., Ercan, M.T., Hincal, A.A., 2000. Scintigraphic imaging of infections with 99m-Tc-labelled glutathione liposomes. *Journal of Microencapsulation* 17, 459–465.
- Fahmy, T.M., Samstein, R.M., Harness, C.C., Mark Saltzman, W., 2005. Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting. *Biomaterials* 26, 5727–5736.
- Faleiros, R.R., Johnson, P.J., Nuovo, G.J., Messer, N.T., Black, S.J., Belknap, J.K., 2011. Laminar leukocyte accumulation in horses with carbohydrate overload-induced laminitis. *Journal of Veterinary Internal Medicine* 25, 107–115.
- Fiske, C.H., Subarrow, Y., 1925. The colorimetric determination of phosphorus. *The Journal of Biological Chemistry* 66, 375–400.
- Hofheinz, R.D., Gnad-Vogt, S.U., Beyer, U., Hochhaus, A., 2005. Liposomal encapsulated anti-cancer drugs. *Anti-Cancer Drugs* 16, 691–707.
- Kullmann, A., Holcombe, S.J., Hurcombe, S.D., Roessner, H.A., Hauptman, J.G., Geor, R.J., Belknap, J., 2014. Prophylactic digital cryotherapy is associated with decreased incidence of laminitis in horses diagnosed with colitis. *Equine Veterinary Journal* 46, 554–559.
- Laverman, P., Brouwers, A.H., Dams, E.T., Oyen, W.J., Storm, G., van Rooijen, N., Corstens, F.H., Boerman, O.C., 2000. Preclinical and clinical evidence for disappearance of long-circulating characteristics of polyethylene glycol liposomes at low lipid dose. *Journal of Pharmacology and Experimental Therapeutics* 293, 996–1001.
- Laverman, P., Dams, E.T., Storm, G., Hafmans, T.G., Croes, H.J., Oyen, W.J., Corstens, F.H., Boerman, O.C., 2001. Microscopic localization of PEG-liposomes in a rat model of focal infection. *Journal of Controlled Release* 75, 347–355.
- Leise, B.S., Faleiros, R.R., Watts, M., Johnson, P.J., Black, S.J., Belknap, J.K., 2011. Laminar inflammatory gene expression in the carbohydrate overload model of equine laminitis. *Equine Veterinary Journal* 43, 54–61.
- Leise, B.S., Watts, M., Tanhoff, E., Johnson, P.J., Black, S.J., Belknap, J.K., 2012. Laminar regulation of STAT1 and STAT3 in black walnut extract and carbohydrate overload induced models of laminitis. *Journal of Veterinary Internal Medicine* 26, 996–1004.
- Levin, V.A., Phuphanich, S., Yung, W.K., Forsyth, P.A., Maestro, R.D., Perry, J.R., Fuller, G.N., Baillet, M., 2006. Randomized, double-blind, placebo-controlled trial of marimastat in glioblastoma multiforme patients following surgery and irradiation. *Journal of Neuro-Oncology* 78, 295–302.
- Longworth, K.E., Jarvis, K.A., Tyler, W.S., Steffey, E.P., Staub, N.C., 1994. Pulmonary intravascular macrophages in horses and ponies. *American Journal of Veterinary Research* 55, 382–388.
- Metselaar, J.M., Wauben, M.H., Wagenaar-Hilbers, J.P., Boerman, O.C., Storm, G., 2003. Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. *Arthritis and Rheumatism* 48, 2059–2066.
- Nourian, A.R., Mills, P.C., Pollitt, C.C., 2010. Development of intraosseous infusion of the distal phalanx to access the foot lamellar circulation in the standing, conscious horse. *The Veterinary Journal* 183, 273–277.
- Obel, N., 1948. *Studies on the Histopathology of Acute Laminitis* (Ph.D. thesis). Almqvist and Wilscells Bottrykeri Ab Uppsala.
- Oyen, W.J., Boerman, O.C., Storm, G., van Bloois, L., Koenders, E.B., Claessens, R.A., Perenboom, R.M., Crommelin, D.J., van der Meer, J.W., Corstens, F.H., 1996. Detecting infection and inflammation with technetium-99m-labeled Stealth liposomes. *Journal of Nuclear Medicine* 37, 1392–1397.
- Parbhakar, O.P., Duke, T., Townsend, H.G., Singh, B., 2005. Depletion of pulmonary intravascular macrophages partially inhibits lipopolysaccharide-induced lung inflammation in horses. *Veterinary Research* 36, 557–569.

- Phillips, W.T., Rudolph, A.S., Goins, B., Timmons, J.H., Klipper, R., Blumhardt, R., 1992. A simple method for producing a technetium-99m-labeled liposome which is stable in vivo. *International Journal of Radiation Applications and Instrumentation. Part B, Nuclear Medicine and Biology* 19, 539–547.
- Pollitt, C.C., 1996. Basement membrane pathology: A feature of acute equine laminitis. *Equine Veterinary Journal* 28, 38–46.
- Pollitt, C.C., Pass, M.A., Pollitt, S., 1998. Batimastat (BB-94) inhibits matrix metalloproteinases of equine laminitis. *Equine Veterinary Journal. Supplement* 119–124.
- Rose, J.S., Neal, J.M., Kopacz, D.J., 2005. Extended-duration analgesia: Update on microspheres and liposomes. *Regional Anesthesia and Pain Medicine* 30, 275–285.
- Rouser, G., Fkeischer, S., Yamamoto, A., 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5, 494–496.
- Sahoo, S.K., Labhasetwar, V., 2003. Nanotech approaches to drug delivery and imaging. *Drug Discovery Today* 8, 1112–1120.
- Szebeni, J., 2005. Complement activation-related pseudoallergy: A new class of drug-induced acute immune toxicity. *Toxicology* 216, 106–121.
- Tadros, E.M., Frank, N., Newkirk, K.M., Donnell, R.L., Horohov, D.W., 2012. Effects of a “two-hit” model of organ damage on the systemic inflammatory response and development of laminitis in horses. *Veterinary Immunology and Immunopathology* 150, 90–100.
- Tsokos, M., 2003. Immunohistochemical detection of sepsis-induced lung injury in human autopsy material. *Legal Medicine (Tokyo, Japan)* 5, 73–86.
- Underwood, C., van Eps, A.W., 2012. Nanomedicine and veterinary science: The reality and the practicality. *The Veterinary Journal* 193, 12–23.
- Underwood, C., van Eps, A.W., Schaer, T.P., Ross, M.W., Storm, G., van Bloois, L., Laverman, P., 2011. A Scintigraphic Comparison of 99mTc Labeled IgG, PEG-Liposome and Leukocyte Accumulation in Experimentally Induced Focal Soft Tissue Infection in the Horse. In: *Proceedings of the American College of Veterinary Internal Medicine Forum*, Denver, CO, pp. 675–676.
- Underwood, C., van Eps, A.W., Ross, M.W., Laverman, P., van Bloois, L., Storm, G., Schaer, T.P., 2012. Intravenous technetium-99m labelled PEG-liposomes in horses: A safety and biodistribution study. *Equine Veterinary Journal* 44, 196–202.
- Underwood, C., Collins, S.N., Mills, P.C., van Eps, A.W., Allavena, R.E., Medina Torres, C.E., Pollitt, C.C., 2015. Regional intravenous limb perfusion compared to systemic intravenous administration for marimastat delivery to equine lamellar tissue. *Journal of Veterinary Pharmacology and Therapeutics* doi:10.1111/jvp.12198.
- van Eps, A.W., 2012. Progress towards effective prevention and therapy for laminitis. *Equine Veterinary Journal* 44, 746–748.
- van Eps, A.W., Pollitt, C.C., 2004. Equine laminitis: Cryotherapy reduces the severity of the acute lesion. *Equine Veterinary Journal* 36, 255–260.
- van Eps, A.W., Pollitt, C.C., 2006. Equine laminitis induced with oligofructose. *Equine Veterinary Journal* 38, 203–208.
- van Eps, A.W., Pollitt, C.C., Underwood, C., Medina-Torres, C.E., Goodwin, W.A., Belknap, J.K., 2014. Continuous digital hypothermia initiated after the onset of lameness prevents lamellar failure in the oligofructose laminitis model. *Equine Veterinary Journal* 46, 625–630.
- Visser, M.B., 2009. Investigation of proteolysis of the basement membrane during the development of equine laminitis (Ph.D. thesis). The University of Queensland.
- Visser, M.B., Pollitt, C.C., 2011. Lamellar leukocyte infiltration and involvement of IL-6 during oligofructose-induced equine laminitis development. *Veterinary Immunology and Immunopathology* 144, 120–128.
- Visser, M.B., Pollitt, C.C., 2012. The timeline of metalloprotease events during oligofructose induced equine laminitis development. *Equine Veterinary Journal* 44, 88–93.
- Wang, L., Pawlak, E., Johnson, P.J., Belknap, J.K., Alfandari, D., Black, S.J., 2012. Effects of cleavage by a disintegrin and metalloproteinase with thrombospondin motifs-4 on gene expression and protein content of versican and aggrecan in the digital laminae of horses with starch gruel-induced laminitis. *American Journal of Veterinary Research* 73, 1047–1056.
- Wang, L., Pawlak, E.A., Johnson, P.J., Belknap, J.K., Eades, S., Stack, S., Cousin, H., Black, S.J., 2013. Impact of laminitis on the canonical Wnt signaling pathway in basal epithelial cells of the equine digital laminae. *PLoS One* 8, e56025.