Intra-articular delivery of a nanocomplex comprising salmon calcitonin, hyaluronic acid, and chitosan using an equine model of joint inflammation

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Abstract

Polyelectrolyte nanoparticle constructs (NPs) comprising salmon calcitonin (sCT), chitosan (CS), and hyaluronic acid (HA) were previously established as having anti-inflammatory potential when injected via the intra-articular (i.a.) route to a mouse model. We attempted to translate the formulation to a large animal model, the lipopolysaccharide (LPS)-stimulated equine model of joint inflammation. The aim was to manufacture under aseptic conditions to produce sterile pyrogen-free NPs, to confirm physicochemical characteristics, and to test toxicity and efficacy in a pilot study. NP dispersions were successfully formulated using pharmaceutical grade source materials and were aseptically manufactured under GMP-simulated conditions in a Grade A modular aseptic processing workstation. The NP formulation had no detectable pathogen or endotoxin contamination. NPs were then tested versus a lactated Ringer's solution control following single i.a. injections to the radiocarpal joints of two groups of four horses pre-treated with LPS, followed by arthrocentesis at set intervals over one week. There was no evidence of treatment-related toxicity over the period. While there were no differences between clinical read-outs of the NP and the control, two synovial fluid-derived biomarkers associated with cartilage turnover revealed a beneficial effect of NPs. In conclusion, NPs comprising

well-known materials were manufactured for an equine i.a.-injectable pilot study and yielded no NP-attributable toxicity. Evidence of NP-associated benefit at the level of secondary endpoints was detected as a result of decreases in synovial fluid inflammatory biomarkers.

Key words: salmon calcitonin, hyaluronic acid, chitosan, joint inflammation, synovitis, nanomedicine, large animal models.

Introduction

Inflammation associated with arthritic conditions is seen in degenerative joint disease and typically occurs in the middle-aged and elderly [1]. Increased cartilage breakdown, bone remodelling, synovial inflammation, and reduction in viscoelasticity of synovial fluid leads to severe pain, as well as joint stiffness and destruction. Increased levels of inflammatory cytokines released from chondrocytes initiate a further increase in the production of IL-1β and collagen-destroying matrix metalloproteinases (MMPs) [2, 3]. Currently available symptomatic treatments for osteoarthritis (OA) include paracetamol, capsaicin, intra-articular (i.a.) corticosteroids, duloxetine, and oral/topical non-

steroidal anti-inflammatory drugs (NSAIDs) [4]. However, the combination of the adverse effects associated with systemic administration of NSAIDs and the short-term and limited pain relief provided by corticosteroids point to the need for disease-modifying OA drugs (DMOADs), as well as regenerative and nanoparticle approaches [5-7].

Sub-cutaneous injections and a nasal spray of salmon calcitonin (sCT) are approved as a second-line treatment for hypercalcemia, osteoporosis and Paget's disease [8]. It was recently tested in Phase III trials as an orally delivered OA treatment to complement existing therapies, although primary endpoints were not met [9]. Part of the mechanism of sCT is thought to be due to prevention of OA-enhanced bone turnover, as well as preservation of the damaged cartilage matrix [10]. Recently, our group demonstrated that sCT, alone and in combination with hyaluronic acid (HA), reduced mRNA expression of key regulators of inflammation, the orphan nuclear receptors, NR4A1-3, as well as of selected MMPs in cultured human chondrocytes, synoviocytes and monocytes [11]. The inhibition of MMP production by sCT also prevented enhanced collagen destruction in OA patient-derived articular explants exposed to TNF-α and oncostatin M [12]. Clinical studies indicate that sCT is analgesic to bone pain in patients with osteoporotic vertebral and distal radius fractures in various formulations via several administration routes [13, 14]. Moreover, a recent clinical trial detected beneficial effects on pain, joint stiffness, and disease activity in the knees of OA patients treated with nasal sCT [15].

While i.a. injections of corticosteroids for OA reduce the risk of adverse effects associated with corticosteroid administered systemically, meta-analysis of clinical trials suggests that efficacy from i.a. injection is quite weak and that pain relief does not last beyond six weeks [16, 17]. Moreover, repeated i.a. administration of corticosteroids is not recommended due to the perceived (albeit rare) risks of septic arthritis [18], along with negative effects on cartilage. While i.a. administration provides an opportunity for highly concentrated local therapy, the administered drug should ideally be slowly released from formulations or implants over several months. Examples include nano- and microparticles, hydrogels, and scaffolds [19-21]. The other approved OA therapy by i.a. administration is the visco-supplement, HA, but its benefits on pain scores are also relatively weak and short-lived [22]. Still, i.a.-injected HA has a good safety profile with only three severe

adverse events detected across over 8000 patients in 41 separate trials [23], and this has encouraged research into how it can be used in combination with other bioactive agents. HA is also being researched as a surface coating of drug-loaded albumin nanoparticles to target the over-expressed CD44 receptor in the chondrocytes of OA-inflamed joints. A 14-day residence time in rat knee joints was achieved for coated particles compared to 7 days for uncoated particles following i.a. administration [24]. By combining HA and sCT with a third component, chitosan (CS), to form a polyelectrolyte complex, we previously saw a reduction of inflammation and preservation of bone and cartilage in the knee joints of the KBxN mouse model of acute inflammatory arthritis following a single i.a. injection [11]. It is this formulation that we attempted to produce in sufficient sterile quantities for i.a. delivery to an equine model.

Joint inflammation is common and naturally expressed in horses [25]. The equine lipopolysaccharide (LPS)-induced transient acute synovitis model allows assessment of therapeutic approaches, as well as measurement of local inflammatory parameters and biomarkers in the synovial fluid and cartilage [26]. A single i.a. injection of 0.5 ng LPS leads to the release of peptide and lipid mediators, as well as an increase in MMP activity and proteoglycan biomarkers in carpal joints within 8 hours, before peaking and eventual reversion to the untreated condition over one week [27]. In response to LPS, increases are detected in white blood cell counts, total protein, and synoviocyte production of prostaglandin E_2 (PGE_2), Substance P, type II collagen cleavage fragments (C2C), and type II collagen carboxy-pro-peptide (CPII) [28]. The equine LPS model has previously revealed anti-inflammatory and analgesic effects of i.a.-administered morphine [29] and orally delivered meloxicam [26] in synovitis, however it has never been used to examine nanoparticle formulations before.

Translating an injectable nanoparticle formulation to the equine model must address manufacturing issues for the injectable nanoparticle product, including ensuring sterility and a reduced risk of pyrogens. In an attempt to scale-up, nanoparticles loaded with curcumin were evaluated for physicochemical properties in comparison to bench-scale [30]. Variables were identified and adjusted during each stage in order to ensure maintenance of physicochemical parameters. A critical requirement for parenteral nanoparticles is a suitable sterilisation technique. Payloads and components of nanoparticle formulations can be susceptible to structural changes caused by gamma irradiation or chemical

treatment of dried nanoparticles, where there is potential loss of stability, and efficacy, and toxicity induction [31, 32]. An additional hurdle for parenteral nanoparticle formulations is the high risk of contamination with pyrogens including bacterial endotoxins [33]. Since removal of endotoxins from nanoparticles is challenging, use of contaminant-free, high grade materials is preferable [34]. Consequently, the primary aim of this study was to assess the safety of a parenteral formulation of the sCT/HA/CS polyelectrolyte complex nanoparticle manufactured for a pilot study for the equine LPS model, while the secondary aim was to test for clinical efficacy and to ascertain if there were any changes in synovial fluid markers representing inflammation, pain and chondro-protection.

Materials and methods

Reagents and chemicals

Materials were of analytical grade and purchased from Sigma-Aldrich (Ireland). Ultrapure, sterile, pyrogen-free materials for use in the equine pilot study were produced according to Good Manufacturing Practice (GMP) guidelines, as well as in accordance with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the International Organization for Standardization (ISO) standards (designated as "pharmaceutical grade material"). Drug Master Files (DMFs) on ultrapure sodium hyaluronate and chitosan salts used in the equine studies are available from NovaMatrix® (Norway).

Formulation of NP polyelectrolyte complexes of sCT, HA, and chitosan

Polyelectrolyte complexed sCT/HA/CS nanoparticles ("NPs") were prepared using either analytical or pharmaceutical grade HA for bench-scale and the equine study, respectively [35, 36]. For the analytical grade process, 0.1 % (w/v) HA solution (sodium hyaluronate from *Streptococcus equi* sp.) was prepared by addition of 100 mg HA to 100 mL deionised water, followed by stirring at room temperature for 12 h, and then by sonication [36]. In the pharmaceutical grade process for the equine study, ultrapure sodium hyaluronate from *Streptococcus*

zooepidemicus (Kibun Food Chemifa Co. LTD, Japan) was dissolved in water-for-injection USP and stirred and sonicated as above. For both syntheses, synthetic sCT (Polypeptide Laboratories, Copenhagen) was then dissolved in the HA solution prior to complexation to give a final concentration of 100 µg/mL sCT. 0.1 % (w/v) chitosan chloride (CS) solution (Protasan® ultrapure CL 113, NovaMatrix™, Norway) was prepared by dissolving 100 mg CS in 100 mL either deionised water or water-for-injection USP. Full details of the materials used in syntheses are given in Table 1. For the bench-scale formulation with analytical grade materials, a 2 mL volume of CS solution was added to 10 mL of sCT/HA solution to yield a mass mixing ratio (MMR) of 5:1 for HA:CS [11] in a beaker with constant stirring at a speed of 1200 rpm. A dispersion of NPs was obtained instantly and stirring was continued for 10 min to enable stabilisation.

For the pharmaceutical grade NP batches used in the equine pilot study, the HA/sCT and CS solutions were prepared under sterile conditions in a laminar air flow cabinet in a pressurised clean room. Both solutions were filtered through 0.2 µm sterile filter units attached to sterile storage containers (Stericup® Millipore, Ireland) [37] to prevent contamination during storage and transfer to the Processing Facility used to fill the vials for the equine study. The sterile solutions were stored at 4 °C and protected from light for a maximum of 24 h at which point they were moved to a Grade A modular aseptic processing workstation (QUBE, Bioquell Ltd, Limerick, Ireland). For NP dispersions used in the equine study, the final formulation volume was 48 mL (with pharmaceutical grade HA and water-for-injection USP), with an MMR of 5:1 for HA: CS maintained as above. Sterile vials were filled with an aliquot of the NP dispersion containing 400 µg of particle-associated sCT (100 µg/mL). The vials were capped and crimped to prevent contamination.

Physicochemical properties of NPs

The mean hydrodynamic diameter of NPs (i.e. particle size, (nm)), the derived count rate (DCR) (kilo counts per second; kcps), and the polydispersity index (PdI) were determined by dynamic light scattering (DLS) at 25 °C using a 173° scattering angle. Zeta potential (ZP) values were calculated from the mean electrophoretic mobility values measured by Laser Doppler Velocimetry (LDV), and by applying the

Smoluchowski equation. DLS and LDV measurements were performed using a Zetasizer Nano series Nano-ZS ZEN3600 fitted with a red laser light beam (λ = 633 nm) (Malvern Instruments Ltd., UK). Samples were placed into a clear disposable zeta cell (DTS1070) for size and ZP measurements. Measurements for each sample were taken in triplicate and values adjusted depending on the dynamic viscosity of the continuous phase. A low frequency vibration viscometer (SV-10 Vibro Viscometer, A&D Company, Limited) was used to measure dynamic viscosity. Viscosities of 0.1 % (w/v) HA solution and NP dispersions were measured following temperature equilibration at 25 °C in a water bath (Reciprocal Shaking Bath Model 25, Precision Scientific Instruments, UK).

Measurement of sCT loading of NPs

Non-associated sCT was separated from NPs using a combined ultrafiltration-centrifugation technique (Amicon Ultra-15, MW cut-off: 50 kDa, Millipore, USA) [11]. A 5 mL sample was centrifuged for 1 h at 3000 rpm in a 5810R centrifuge (Eppendorf, UK) at 20 °C. After centrifugation, the volume of filtrate was measured, and the filtrate was assayed for sCT content by HPLC (see below). This quantity of sCT was referred to as "non-associated" or free sCT. The NP dispersion in the sample reservoir ("concentrate") was standardised to 5 ml with deionised water and was assayed for sCT content, ("associated sCT"). Both aliquots were compared for mass balance. An aliquot of the concentrate was analysed for signs of disintegration of NP by measuring the mean particle size, PdI and ZP and compared to values before the centrifugation-ultrafiltration steps. The association efficiency was calculated using the following equation:

(Equation 1) $$Association\ efficiency\ (\%) = \frac{Total\ sCT - Free\ sCT}{Total\ sCT} * 100$$

Where $Total\ sCT$ was the total sCT concentration in the formulation, and $Free\ sCT$ was determined from the filtrate by RP-HPLC. The sCT loading was calculated by:

(Equation 2) $$sCT\ loading\ (\%) = \frac{Total\ sCT - Free\ sCT}{Total\ Ingredients} * 100$$

Where $Total\ sCT$ and $Free\ sCT$ were the same as above, while $Total\ Ingredients$ was the sum of all the materials in the formulation.

***In vitro* release of sCT from NPs and quantification by RP-HPLC**

For release studies, 4.5 mL of the NP dispersion were suspended in 13 mL PBS with 0.02 % (w/v) sodium azide to prevent microbial degradation of sCT [38]. The release study was performed at 37 °C with horizontal shaking (60 rpm) (reciprocal Shaking Bath Model 25, Precision Scientific, UK). At each time point (0, 1, 2, 4, and 6 h), 1 mL aliquots were collected and replaced with fresh PBS to maintain sink conditions. The aliquot was placed in the sample reservoir of a centrifuge (Amicon Ultra-2 Centrifugal Filter Unit with Ultracell-100 membrane, MW cut-off 100 kDa; Millipore, USA), centrifuged at 4500 rpm for 20 min at 20 °C in order to separate the released sCT from NPs. The filtrate was assayed for sCT content and release profiles were constructed. Reverse phase HPLC was used to determine sCT concentration [39]. A HPLC system (Waters, USA) was equipped with a binary HPLC pump system (Model 1525), an auto-sampler (model 717plus), and a dual λ absorbance detector (Model 2487). Chromatographic separations were carried out with a C_{18}, 15 – 20 µm, 3.9 x 300 mm column (µBondapak, Waters, Ireland). Measurements were conducted by injecting 50 µL of sample or standard (0.1 – 100 µg/mL sCT in mobile phase) and isocratic elution was carried out at a flow rate of 1.5 mL/min. The mobile phase was composed of 64 % (v/v) of aqueous phase (1.8 g/L NaCl, 0.05 % (v/v) trifluoroacetic acid in water) and 36 % (v/v) of acetonitrile (Chromasolv® gradient grade for HPLC ≥ 99.9 %). Absorbance was measured at 214 nm. Data was collected and processed using Breeze™ software, Version 3.30 SPA (Waters, USA).

Sterilisation and de-pyrogenation of materials for the equine study

All glassware, spatulas, scissors, stoppers and stirrer bars used during NP preparation and storage were either purchased in pre-sterilised endotoxin-free form or soaked in an alkaline detergent (E-TOXA-CLEAN; Sigma-Aldrich, USA) to remove contaminants. Stoppers for volumetric flasks and stirrer bars were autoclaved for 20 min at 121 °C (Autoclave; Astell Scientific, UK). The autoclaving process was validated using biological indicators (Mesa ProTest Steam, *G. stearothermophilus* ATCC® 7953™ Log 6) according to the user manual. All glassware and metallic items were heat-sterilised and all equipment was wrapped in tin foil and kept at 250 °C for at least 2 h. To validate heat-sterilisation, water-for-injection was incubated in a heat-sterilised beaker and assessed for endotoxin content. All technical equipment including the probe sonicator and stirrer plate were bagged and sprayed down using 70 % (v/v) isopropanol (T.E. laboratories, Ireland) before transferring it into a laminar air flow cabinet (LAF). The aseptic environment in the modular aseptic isolator, the QUBE, was obtained using hydrogen peroxide vapour. Prior to the preparation process, all items and surfaces of the QUBE were sprayed with 70 % (v/v) isopropanol and set up for the gas cycle in the isolator.

Compliance with aseptic conditions throughout NP production for equine study

During the production of NPs in the QUBE, both non-viable and viable bacterial counts were monitored inside the isolator. The non-viable counts were monitored using a particle counter (integrated in the isolator) and the viable counts were monitored using settle plates (Merck, Ireland) and a viable air sampler (integrated in the isolator). A surface sample was also taken at the end of the study using a contact plate (VWR Chemicals, Ireland). All environmental monitoring plates (settle, viable and contact) were incubated at 25 °C for 7 days to check for fungus and yeast, followed by incubation at 35 °C for a further 7 days to check for bacteria.

Validation of sterility of polymer solutions and final NP product

Sterility testing was carried out under aerobic and anaerobic conditions to check for the presence of facultative bacteria. Assays were based on the direct inoculation technique described in the European Pharmacopoeia [40]. The specified quantity of sample under test was drawn aseptically from the containers and added to 10 mL of Tryptone Soya Broth (TSB, Oxoid™, UK) and incubated at 37 °C for 18 h. The broth was then sub-cultured onto Columbia blood agar plates (Oxoid™, UK), which were then incubated under aerobic and anaerobic conditions at 37 °C for up to 36 h. The plates were checked for the presence of bacteria to determine the sterility status of the samples. The TSB was also inoculated onto Sabauroud Dextrose agar (Oxoid™, UK) to check for fungal growth. These plates were incubated at 37 °C for 5 days. In tandem, a further 2 mL of the samples was added to Schaedler Anaerobic broth (Oxoid™, UK) to check for the presence of strict anaerobes. This broth was incubated for up to 36 h in a gas jar at 37 °C under anaerobic conditions. Next, the broth was sub-cultured onto Columbia Blood agar and the plates were placed into a gas jar and incubated at 37 °C for up to 36 h.

Semi-quantitative determination of endotoxin levels

To determine the level of endotoxins in the sample, a semi-quantitative gel clot *Limulus* amebocyte lysate (LAL) method was used (E-TOXATE™ Kit, Sigma-Aldrich, USA) [41]. All materials used were labelled as sterile and pyrogen-free by the manufacturers. Endotoxin detection was carried out according to the Sigma-Aldrich Technical Bulletin for E-TOXATE™ Kits (ET0100, ET0200 and ET0300), based on guidelines from the European Pharmacopeia on the “test of bacterial endotoxins (LAL Test)” [42]. 0.1 % (w/v) dispersions of NPs as well as 0.1 % (w/v) polymer solutions were tested. Samples were transferred to pyrogen-free polystyrene cell culture tubes with a 2-position vent stopper (12.4 x 75 mm; Greiner, Austria). Just before the assay, pH of samples was adjusted to a value between pH 6 – 8 with endotoxin-free 0.1 N HCl or NaOH. The standard curve was generated by assaying known amounts of endotoxin in water-for-injections. The threshold of detection (assay sensitivity) was 0.05 – 0.1 EU/mL and was confirmed for all assay lots used. To further validate the results, controls to

account for possible inhibition of the assay were included by spiking all samples tested with endotoxin. Samples prepared under sterile conditions were compared to non-sterile samples.

In vivo pilot study: i.a. injection of NPs into LPS-stimulated radiocarpal equine joints

The equine pilot study was approved by the UCD Animal Research Ethics Committee and performed under licence from the Irish Health Products and Regulatory Authority (Project Authorisation # AE18982/P081). Eight healthy, mature mares (age 12.3 ± 5.3 years, height 158 ± 4.4 cm, weight 567.6 ± 72.5 kg) from the research herd were brought indoors in separate box stalls and were fed 1 kg of maintenance concentrates B.I.D. with hay *ad libitum*. The horses had clinically and radiographically normal radiocarpal joints. Synovitis induction and all other procedures were performed as previously described with minor changes [27]. LPS from *Escherichia coli* O55:B5 was diluted to a final concentration of 0.63 ng/mL in sterile lactated Ringer's solution. Sterile arthrocentesis of the radiocarpal joint was performed with a 20 G x 3.8 cm needle. Synovial fluid at t = 0 h was withdrawn for analysis and 0.8 mL of LPS solution was then injected to a randomly assigned left or right radiocarpal joint. The target dose of sCT required for the equine radiocarpal joint was estimated according to several assumptions including an association efficiency of < 100 %. In [11], the sCT dose administered to the KBxN mouse knee was 0.2 µg in a maximum joint volume of 5 µL (i.e. 40 µg/mL). The equine radiocarpal joint volume contains a volume of 12.6 ± 1.5 mL [43], a 2000 fold dilution compared to mouse, so the estimated equine dose target was 0.4 mg sCT per joint, ultimately achieved by injecting a volume of ~4 mL (0.1 mg/mL). The NP dispersion was injected to a group of four horses 120 min after the LPS injection, while four horses received the lactated Ringer's solution (vehicle) control. Arthrocentesis resulted in an extraction of approximately 4 – 5 mL from each injected joint at t = 2, 8, 24 and 168 h.

Clinical measures of NP safety and efficacy in a pilot equine study

Part of the synovial fluid aspirate was placed in EDTA tubes for white blood cell count determination by haemotocytometry and total protein measurement using a refractometer. The remaining synovial fluid was centrifuged for 15 min at 4 °C and 3500 rpm. Supernatants were immediately frozen and stored at -80 °C. Additionally, every 2 h between t = 0 – 8 h and at t = 24, 48, 72, 96, 120, 144 and 168 h, each horse's behaviour, temperature, pulse and respiratory rate was recorded. Lameness was scored on a standardised 0 – 5 scale [44], and the joint was palpated to establish a subjective effusion score ranging from 0 – 4 (1 = mild, 2 = moderate and 3 = severe joint effusion, 4 = severe swelling of the entire carpal region). Carpal circumference was measured with a tape measure. The horses were regularly assessed by a veterinarian for signs of potential NP toxicity in respect of overall health over the one week period. These adhered to a general distress scoresheet established for the model (Suppl. Table 1).

Measurement of synovial fluid biomarkers: C2C, CPII, PGE_2 and Substance P

Equine synovial fluid was analysed for collagen-Type II cleavage protein (C2C) by ELISA (Ibex Pharmaceuticals Inc., Canada) according to the manufacturer's instructions. In brief, synovial fluid samples were stored at -80 °C before use and thawed on ice. 50 µL of synovial fluid diluted in Buffer III (1:2.5) and 50 µL C2C Antibody in Assay Buffer were pipetted into each well of a polypropylene mixing plate. This was pre-incubated for 30 min at 20 – 25 °C on an orbital plate shaker at 600 – 700 rpm (Titramax 1000, Inkubator 1000, Heidolph, Germany). 80 µL from each well were transferred to the ELISA plate and incubated for 1 h at 20 – 25 °C on a plate shaker at 600 – 700 rpm. The plate was washed three times with the wash buffer provided with the kit. 100 µL of goat anti-mouse horseradish peroxidase conjugate in Buffer III were added to each well and incubated for another 30 min at 20 – 25 °C on a plate shaker at 600 – 700 rpm followed by washing as above. 100 µL 3,3',5,5'-tetramethylbenzidine substrate was added to each well and incubated for 30 min (20 – 25 °C, 600 – 700 rpm shaking). 100 µL stop solution were added to each well and the plate was read at 450 nm. Standards were included on each plate and GraphPad Prism® 5 software (San Diego, USA) was used to calculate values of unknowns from the standard curve. Synovial fluid samples were also analysed for collagen Type II (CPII), prostaglandin E_2 (PGE_2) and Substance P by ELISA. The ELISA kits were from Ibex Pharmaceuticals) for CPII, and from

Cayman Chemicals (Ann Arbor, MI, USA) for PGE_2 and Substance P. Procedures were carried out according to the manufacturer's instructions using similar procedures as above.

Statistical analysis

Statistical analysis for all experiments was carried out using GraphPad Prism® 5 Software. Regarding studies comparing viscosity of analytical grade and pharmaceutical grade HA, statistical differences between groups were determined by two-way ANOVA followed by Bonferroni's post-test. Statistical differences for other experiments with normally distributed data were determined using Student's unpaired t-test. Differences were considered significant if $P<0.05$.

Results

Comparison of properties of NPs formulated at bench-scale and for equine study

The major challenge in designing NPs for a large animal study is to produce sufficient material under aseptic processing conditions to produce a sterile, endotoxin-free product. In addition, similar peptide loading and NP physicochemical characteristics that have been optimised during initial studies must be maintained upon increasing the yield of NP required to dose a large animal. The MW of pharmaceutical grade HA was lower than that of analytical grade HA (Table 1). Therefore, pharmaceutical grade HA was sonicated and the required sonication time was related to dynamic viscosity and compared to data on analytical grade HA from published literature [35]. Following sonication, the difference in dynamic viscosity between pharmaceutical and analytical grade HA became insignificant due the levelling off in depolymerisation [45]. For the pharmaceutical grade HA, the sonication period was optimised at 120 min (Table 2). Mean particle size, PdI and ZP values for bench-scale NPs made with both grades of HA were within a similar range to those previously reported in the KBxN mouse study [11] (Table 3). When moving from the 12 mL volume to the 48 mL volume batches of NPs using pharmaceutical grade HA, the particle size and PdI values

were unchanged, although the ZP values were statistically more negative and the dynamic viscosity values were statistically higher. In sum, according to the DLS and viscosity measurements, the NPs formulated for the equine study had a mean size of 190 nm, a low PdI of 0.17, a negative ZP of -42 mV, and a dynamic viscosity of 1.14 mPa*s.

sCT association efficiency, loading, and *in vitro* release from NPs

A centrifugation-ultrafiltration method separated free sCT from NP-entrapped sCT and allowed calculation of the association efficiency (AE) and peptide (sCT) loading (PL). To confirm that this method was valid, 0.1 mM NaOH was added to NP dispersions made from analytical grade materials in equal volumes designed to release sCT [36]. After isolation of sCT associated with NPs by centrifugal ultrafiltration, the sCT concentration in the sample was measured by HPLC before and after addition of NaOH. As the concentration of sCT detected in NPs without addition of NaOH (122 ± 45 µg/mL; n = 5) was not different to the concentration of sCT detected after exposing them to NaOH (117 ± 35 µg/mL; n = 5), it was concluded that dissociation of NPs in the HPLC mobile phase had occurred. In the centrifugation-ultrafiltration method, sCT associated with the particles was in the concentrate while free sCT was in the filtrate. Table 4 shows the AE and PL for concentrates using analytical versus pharmaceutical grade materials (bench-scale and equine study respectively): both groups of NPs had high AE values > 90 %, while the PL was 7 – 8 %. These data indicated retention of the main features of the NP dispersions produced with pharmaceutical grade materials for the equine pilot study. The release of sCT from NPs made from pharmaceutical grade HA (MMR 5, 100 µg/mL sCT loading) and designed for the equine study was assessed in PBS (Fig. 1). Please note, the comparison with a control formulation was not possible as all three components are required for the particle formation. A volume of NP dispersion was mixed with 13 mL of PBS, similar to that reported for equine radiocarpal synovial fluid [43]. Up to 54 % of the initial sCT concentration was released from NPs in 60 min, followed by gradually decreasing release over a further 5 h. Up to 88 % of the initial sCT concentration was released after 6 h. These findings are similar to *in vitro* sCT release data from bench-scale NPs made from analytical grade HA used for studies in mice [11].

Assessment of aseptic processing and endotoxin levels in NPs used for the equine study

In addition to using pharmaceutical grade base material, aseptic processing was put in place to avoid contamination of the formulation during production. Components and the final product were assessed for sterility and endotoxin levels. *Geobacillus stearothermophilus* spores were used to validate the steam sterilisation cycle. The biological indicators exposed to steam sterilisation did not exhibit a colour change (Fig. 2), therefore validating successful steam sterilisation of stoppers and magnetic stirrers. Continuous particle and microbiological monitoring during NP production and vial filling was performed in the QUBE. Non-viable particle count, monitored within the isolator after air clean up using HPV, met E.U. regulatory criteria during manufacturing. Example of agar plates used for microbiological monitoring are shown in Suppl. Fig. 1. No growth was seen in settle plates, active viable air sampling plates, or contact plates. Therefore, also the microbiological monitoring met the E.U. criteria of <1 CFU/plate for a Grade A environment [46]. Additionally, the final NP products were assessed for bacterial and fungal growth. No aerobe, anaerobe or fungal growth was seen using Tryptone Soya broth except for one sample from the first batch, which tested positive for alpha-haemolytic *Streptococcus* species. In addition, one sample out of 30 samples showed evidence of *Staphylococcus* growth in Schaedler anaerobic broth, thus just two samples from two batches out of the total of six batches made did not comply with sterility standards (Table 5).

Endotoxin measurements in materials used in the scaled up NP process

Endotoxin levels in polymer solutions, NP dispersions, and de-pyrogenated glassware were determined. The sensitivity of the LAL gel clot assay (0.05 – 0.1 EU/mL) was confirmed for all assay lots used. In order to eliminate the possibility of false negatives and therefore ensure compatibility of the assay with the samples, polymer solutions and NPs made from analytical grade polymers as well as endotoxin-spiked pharmaceutical grade samples were tested first. Levels below the assay sensitivity were observed in pharmaceutical grade samples prepared for *in vivo* studies (Table 6). On the other hand, no inhibition of the gel clot assay was detected when spiking pharmaceutical grade samples with endotoxins. The importance of using pharmaceutical grade materials for the NPs for equine studies was confirmed by the recording of

endotoxin-positive readings when NPs were formulated using non-sterile, analytical grade polymers. The effectiveness of de-pyrogenation of glassware by heat sterilisation was also assessed using the LAL assay. A glass beaker of each batch of heat-sterilised glassware was incubated with water-for-injection under aseptic conditions for 4 h (the time needed to prepare polymer solutions). Endotoxin levels below the assay sensitivity level were confirmed by the absence of gelling of the sample. The combination of pharmaceutical grade material, heat sterilisation, and the aseptic work environment therefore ensured negligible endotoxin levels throughout the scaled up NP process.

Clinical measures from the equine pilot study

Safety and efficacy of radiocarpal joint injection of NPs was tested versus vehicle control following administration 2 h after the LPS challenge. Regarding efficacy, there was no statistical differences in white blood cell count (Fig. 3A), total protein (Fig. 3B), effusion score (Fig. 3C), lameness (Fig. 3D), or radiocarpal circumference (Fig. 3E) between the two groups. In terms of the primary efficacy, although there was no suggestion of a clinical benefit for the NP, the study was a pilot and not powered to examine this statistically. In terms of toxicity, there were no events that required treatments, euthanasia, or study termination. There were no adverse events in either group and no changes in equine temperature or heart rate at the selected time points out to 168 h. The general distress score was also not different between the groups and these data incorporate temperature and heart rate changes over time (Fig. 3F).

Biomarkers from the synovial fluid from NP-injected radiocarpal joints

A comparison was made between LPS followed by lactated Ringer's solution controls versus LPS followed by NPs in equine synovial samples over 168 h for two inflammatory markers, PGE_2 and Substance P, and two markers of cartilage metabolism, C2C and CPII. In the controls, there were significant increases in all four molecules, PGE_2 (8 h), C2C (24 h), CPII (24 h) and substance P (8 h) compared to 0 h, which returned to near basal values by 168 h (Fig. 4A-D). As shown in Fig. 4A, addition of NPs significantly eliminated the increase in PGE_2 values at 8 h observed in controls. Furthermore, the addition of NPs significantly attenuated LPS induced CPII levels compared to controls

(Fig. 4C). No statistical changes were observed comparing controls to NPs with regard C2C or Substance P at the time points chosen (Fig. 4B and D). Overall, while the clinical data did not indicate differences between the groups, biochemistry analysis indicated that the NPs curtailed the LPS-induced responses of two important biomarkers in synovial fluid at the 8 h and 24 h time points for PGE_2 and CPII respectively.

Discussion

There is frustration at the relatively slow rate of translation of nanomedicine technologies to market [47]. Although synthesising nanoparticle formulations in sufficient quantities for large animal studies and human clinical trials poses technical and logistical challenges [48], it is necessary to put effort into solving manufacturing issues early in the project in order to assess translational potential. Thus, moving to large animal studies subsequent to achieving successful NP data in rodent models should be a high priority. In order to maximise translation potential, Lakkireddy *et al*. [49] have proposed quality management principles for developing nanomedicines and advocate use of approved raw materials, simple assembly processes for scale-up, along with thorough *in vitro* characterisation of the final product ahead of *in vivo* testing. We attempted to translate anti-inflammatory NPs from our previous study in which proof-of-principle was first achieved in the KBxN inflammatory mouse model following a single i.a. administration [11]. Here, the NPs were produced in sufficient quantities using pharmaceutical grade reagents and with an aseptic manufacturing process designed to ensure a pyrogen-free, sterile product for administration to an equine LPS joint inflammation model. The overall outcomes of the current study were clear in respect of the maintenance of equivalent NP physicochemical characteristics to the original formulation, the lack of contamination of the final parenteral product, and qualitative descriptive indications of safety in four horses as part of a pilot study. Although there were no clinical indications of efficacy according to the five parameters assessed, there were relative reductions in the levels of two important synovial fluid markers at 8 h and 24 h, namely PGE_2 and CPII, which are regarded as being important in cartilage turnover (CPII) [50] and the inflammatory process (PGE_2) [51].

The first challenge to address in the study included ensuring that the selected materials were of the highest commercially available quality.

Materials used in the production of parenteral formulations must adhere to standards, e.g. bacterial, fungal and endotoxin load, established by pharmacopeia (USP, BP, Ph. Eur.) in order to reduce the possibility of adverse events following parenteral administration in *in vivo* large animal studies. This was achieved by changing from analytical grade HA material previously used in the mouse study to pharmaceutical grade material. Furthermore, analytical grade deionised water was replaced with pharmaceutical grade water-for-injection, which was sterile and endotoxin-free. In making the substitution of HA and water-for-injection as components of the NPs designed for the equine study over the bench-scale version of the NP dispersion (while maintaining an HA: CS MMR = 5), there was a possibility that the physicochemical properties might be altered. Changing the polymer grade or volume of the formulation can alter peptide structure and conformation and can induce crosslinking and degradation, thereby resulting in reduction in efficacy and/or increased toxicity [47, 52]. Thus, understanding of the relationship between the physicochemical features of the NPs and biopharmaceutical performance are essential. In respect of standardising the physicochemical features for NPs used in different studies, formulating particles with similar physicochemical features when upgrading reagents was achieved in the current study, as the most important parameters of particle size and size distribution of the final sterile non-pyrogenic NP dispersions in a 48 mL volume for the equine study were not different from those made at 12 mL bench-scale volume, with both made with pharmaceutical grade HA. It is important to emphasise that this was not a scale-up study *per se*: the numbers of horses in the pilot study were low, so we simply calculated a batch size to cater for the synovial fluid volume based on estimating the requirement for mouse-to-horse. Nonetheless, a scale-up process for horses or humans should ultimately be possible as it is a one-pot synthesis, uses approved materials of GMP grade, and can meet safety criteria for a parenteral injection.

Our main concern was that the NPs could cause toxicity and/or major adverse events in the horse. Little is known about the possibility of equine hypersensitivity in response to i.a.-implanted biomaterials, but horses have been seen to respond with substantial more inflammation than other large animal species towards i.a.-delivered biomaterials and implants, suggesting that the potential risks from NPs might be high in this species (Brama, P., personal communication). Others recognise the importance of the unique aspects of safety testing in large animal models and have, in contrast, used particulates to attenuate local toxicity of co-administered implants. For example, polylactide-co(glycolide)

(PLG) microparticles containing corticosteroids formed a hydrogel coating and prevented the foreign body reaction to subcutaneously-implanted silicon chips in Goettingen mini-pigs [53]. Implanted particulates thus have the potential to both generate unforeseen toxicity in large animals, as well as having capacity to offset toxicity of associated implanted materials.

We formulated a sterile uncontaminated non-pyrogenic parenteral anti-inflammatory NP product for testing in an equine model. Although the NP had quite a low potential for inducing toxicity as the components are either approved or have a long history of use in man, a range of approaches were used in order to avoid the introduction of contaminants into the parenteral formulation. The European Pharmacopoeia defines aseptic processing as: "*maintaining the sterility of a product that is assembled from components, each of which have been sterilised. This may include (…), aseptic blending of formulations followed by aseptic filling and aseptic packaging*" [54]. Therefore, NPs were formulated under aseptic conditions and were then processed in a GMP-simulated operational manufacturing laboratory. To ensure constant monitoring of the environment during production and vial-filling, the final product as well as the polymer solutions were tested for microbial and fungal growth. After one sample was contaminated by a *Staphylococcus* strain and one other sample by a *Streptococcus* strain, the process of aseptic production was further investigated, however no bacterial or fungal growth was observed in any other samples using that process, so the conclusion was that contamination during sample transfer for sterility validation was the likely source. A group at the MD Anderson Cancer Centre has established a GMP-manufacturing site for liposomes for cancer clinical trials and concluded that high level quality control and quality assurance was vital [55]. Large-scale manufacturing of GMP-compliant doxorubicin-loaded anti-EGFR-immuno-liposomes for human cancer trials has also been achieved [56]. In that study, liposomes were manufactured in a clean room according to GMP-guidelines and compliance of the formulation process as well as product analysis and sterility were confirmed.

The European Pharmacopoeia lists three methodologies for the quantification of bacterial endotoxins by LAL-assay: (i) gel-clot technique, (ii) turbidimetric technique or (iii) chromogenic technique [42]. A semi-quantitative gel-clot assay was selected for the current study. The acceptable endotoxin limit for parenteral formulations was calculated by the K/M formula (K: maximum allowable endotoxin exposure, 5

EU/kg/h; M: maximum recommended human dose of product per kg/h); it has been extrapolated to various animal species [57]. In order to avoid interference with the LPS-induced equine joint inflammation model, we needed to omit additional bacterial endotoxins. The labelled lysate sensitivity of the kit was confirmed by testing standard solutions. All samples and standards were tested in glass tubes since leachables from plastic tubes can interfere with the LAL assay [58]. The pH of each sample was adjusted to a value of 6.0 – 8.0 to provide a pH optimum for serine proteases as they play a pivotal role in the LAL reaction [58]. Phosphate groups on the glycosidic portion of lipid A, a component of LPS, can also be electrostatically attracted to cationic nanoparticles or with divalent cations causing assay interference [58, 59]. Analytical grade samples as well as LPS-spiked samples tested positive, ruling out binding of LPS to the surface of the NP. On the other hand, by binding divalent cations and reducing the aggregation state of LPS, chelating agents can increase the reactivity of LAL assays, yielding false positive data [58]. However, as the pharmaceutical grade material tested negative, an increase in assay sensitivity induced by the samples did not occur. Results from sterility and endotoxin tests were further confirmed by the lack of adverse effects related to NP contamination *in vivo*.

Studies using nanoparticles and microparticles entrapping therapeutics have shown inflammation-reducing effects following i.a. injection by retention in preclinical animal joints based on particle diameter [60]. There is debate however, concerning the ideal particle diameter for joint retention following i.a. delivery and this is further confounded by the influence of particle composition, particle shape, and peculiarities of the selected animal model. Thus, the literature advocates a wide range of specifications with no consensus to date [61-64]. For i.a. delivery, the particle diameter of our NPs might not be as important as their sustained release properties. The cartilaginous extracellular matrix is permeable to smaller molecules than albumin (MW 67 kDa) depending on charge and conformation, and consequently should not prevent particle-released sCT from diffusing into cartilage. However, once in the joint space, macromolecules are easily removed via the lymphatic system independent of particle size. Higher synovial permeability and enhanced drainage from the joint space in patients with inflamed joints might further increase removal [61, 65]. Thus, the encapsulation of sCT in a slow-releasing nanoparticle matrix targeting chondrocytes might be beneficial for a prolonged retention of the drug at the site of injection [66].

The equine LPS joint inflammation model has already been used to screen efficacy of anti-inflammatory [26] and opiate [29] molecules, however it has never been used to assess nanoparticle constructs. High variability was detected in the clinical read-outs. Lasarzik *et al*. [67] argue that white blood cell counts and total protein assay in synovial fluid have a large range of values and may not discriminate healthy and mildly arthritic joints. Indeed, no differences in lameness and effusion scores nor in carpal circumference were noted following administration of NP dispersions, albeit with low numbers of animals, so the model will need to be further assessed to see if it has sufficient discriminatory power to rank order therapeutic and regenerative interventions or if it is limited to qualitative conclusions on safety and efficacy. Repeated arthrocentesis *per se* is not thought to contribute to synovial joint cytology in healthy horses with normal joints [68], so it is unlikely to have contributed to the inflammation-inducing effects of LPS. Another modification to improve reproducibility and to screen for efficacy will be to allow each horse serve as its own control by administration of LPS and NPs in pair-wise radiocarpal joints and this is planned for future iterations in studies designed with power to calculate efficacy.

Regarding study limitations there are several. Firstly, the LPS model is an acute inflammation model (as is the KBxN mouse model in which initial efficacy was obtained [11]) and neither recapitulates all the inflammatory features of degenerative joint disease. Secondly, the equine pilot study only compared NP versus vehicle controls with low numbers of subjects; a full study will compare the NP against both a positive control (e.g. a glucocorticoid, triamcinolone), a negative control (vehicle) and with larger numbers of subjects in a three-way comparison. Thirdly, the anti-inflammatory data was only significant in terms of the synovial fluid markers and not in the overall clinical benefit, and this reflects most likely on the fact that the construct was not optimised in terms of controlling the sCT release rate towards an ideal target profile of three-six months. While the release profile over hours rather than days proved adequate for the time scale of the KBxN mouse study [11], in retrospect it was clearly not optimal for the equine pilot study. Further work with coated- or lipid-based nanoparticle compositions designed for slow release in equine synovial fluid medium *in vitro* will likely lead to superior constructs that can release over longer periods in the joint. In addition, comprehensive studies will need to assess the biochemical changes in the synovial fluid at intervening time points between

24 and 168 h. Fourthly, retention of nanoparticles in the joint space needs to be established using fluorescent imaging of labelled payload in labelled particles, where it is possible to assess isolated equine limbs *post mortem*. No doubt the equine model will offer a perspective on optimal particle characteristics for joint retention that will differ from that advocated for rodents and other preclinical models. Finally, we recognise that the range of inflammatory markers examined was somewhat limited, albeit selectively covering inflammation, pain and cartilage turnover/chondroprotection. Future studies will need to examine comprehensive expression of synovial MMPs, equine NR4A1-3, glucose-aminoglycans (GAGs), and multiplex cytokines in the event that clinical efficacy is met. In addition, fine-tuning the LPS potency and the dose level is needed to standardise the challenge in order to allow comparisons between studies testing efficacy of nanoparticle prototypes.

Conclusions

This study provided a roadmap of how to translate an i.a. nanoparticle formulation from a murine inflammatory model to a large animal model. The formulation was adapted as an aseptic GMP-simulated process using pharmaceutical grade and pure source materials. The use of filtration sterilisation and clean-room vial-filling ensured a product that was pathogen- and pyrogen-free. In the pilot study, there were no indications of NP-attributable adverse events. Although clinical measures of efficacy in response to the NPs were not apparent, reduction in two synovial markers of inflammation and cartilage metabolism suggested subtle beneficial effects. Thus, the equine LPS inflammatory model may be useful to assess optimised versions of these and other i.a.-injectable anti-inflammatory nanoparticles, which show promise in rodent studies.

Acknowledgements

This study was supported by the Science Foundation Ireland (SFI) Centre for Medical Devices (CURAM) (Grant # 13/RC/2073), the SFI Synthesis and Solid State Pharmaceutical Centre (Grant # 12/RC/2275), by the Wellcome Trust Institutional Strategic Support Fund Clinician

Stimulus Scheme (CK), and by the European Union Regional Development Fund. Thanks also to Shada Warreth and Aoife Barron at the National Institute of Bioprocessing and Research Training, Dublin, for assistance with manufacturing of the NP formulation under GMP-simulated conditions. Thanks also to Dr. Nola Leonard of the UCD School of Veterinary Medicine for microbiology assay advice. Thanks to Pixabay.com for the download of the image used in part of the graphical abstract.

Conflict of interest

All authors have declared no conflict of interest. The equine experiments comply with the current laws of Ireland. All institutional and national guidelines for the care and use of laboratory animals were followed.

Figure Captions

Fig. 1. Release profile of sCT from NPs formulated with sCT, HA and CS, and made from pharmaceutical grade materials in PBS at pH 7.4 at 37°C. Mean ± SD (n = 3).

Fig. 2. Validation of autoclaving process. Lack of colour change in sterilised vials after incubation at 60 °C for 24 h confirms an effective autoclaving process. Autoclaved equipment was safe to use for the production process of the formulation injected in *in vivo* studies.

Fig. 3. Clinical parameters from the equine pilot study. For all graphs, the dashed line with closed circle represents the horses that received LPS and NPs; the solid line with open circle represents the horses that received LPS and sterile lactated Ringer's solution (control). A. WBCC, white blood cell count (cells x 10^9/L); B. total protein (g/L); C. effusion score; D. change in lameness grade; E. change in carpal

circumference (expressed as a ratio of the carpal circumference (cm) at each time point: carpal circumference (cm) at 0 h; F. general distress score (according to criteria in Suppl. Table 1). Values are given as mean ± SD at each time point (n = 4). WBCC values were omitted for one horse at 2 h due to clotting of the sample. The x-axis is drawn on a non-linear scale.

Fig. 4. NPs attenuated biochemical markers of joint damage in an LPS equine model of joint disease. At time zero, 8 mares received an i.a. injection of 0.5 ng/mL LPS into the radiocarpal joint. At 2 h post LPS, 4 horses received a single i.a. injection of NPs containing 0.4 mg sCT/joint, while 4 received lactated Ringer's solution as a negative control. At time 0, 2 h post LPS, 8 h post LPS, 24 h post LPS and 168 h post LPS, synovial fluid was extracted and analysed for markers by ELISA. (A) PGE_2, (B) C2C, (C) CPII and (D) Substance P. The y-axis is split in part A in order to be able to view the actual raw values at times 0, 2 , 24 and 168 h. Data are expressed as pg/mL or ng/mL values ± SEM (n = 4 replicates per group). * P<0.05; ** P<0.01, *** P<0.01; NS: Not Significant.

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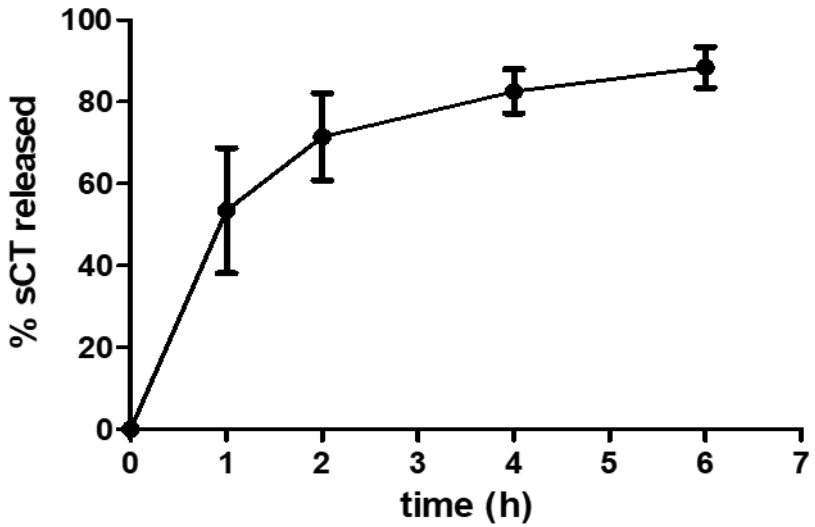

Fig. 1

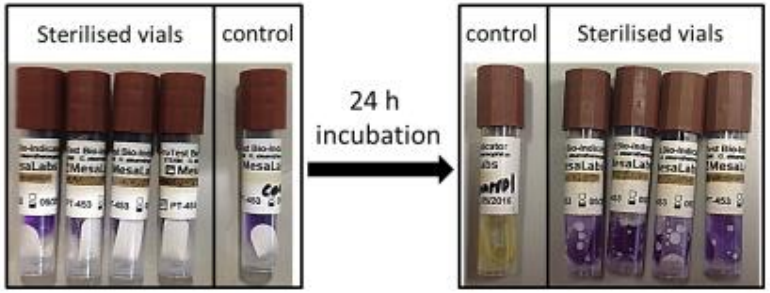
Sterilised vials
control
24 h
incubation
control
Sterilised vials

Fig. 2

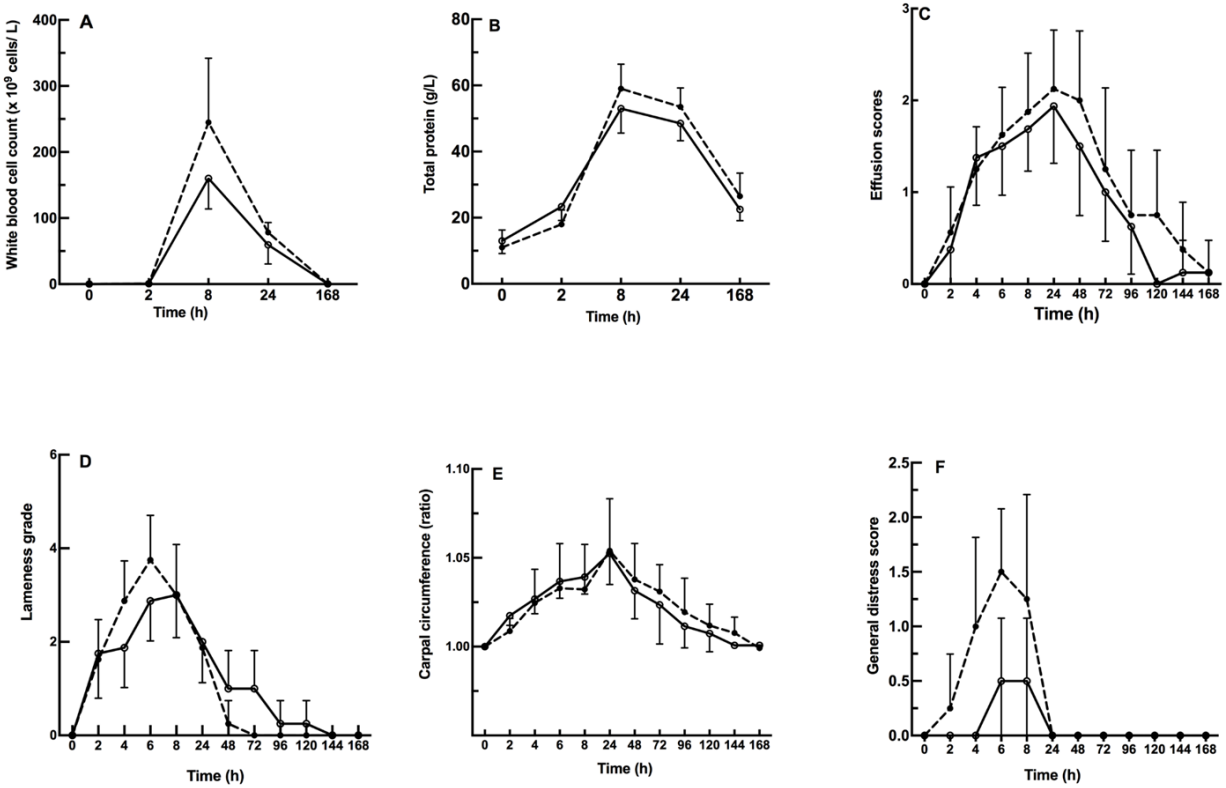
A
White blood cell count (x 10^9 cells/ L)
400
300
200
100
0
0
2
8
24
168
Time (h)
B
Total protein (g/L)
80
60
40
20
0
0
2
8
24
168
Time (h)
C
Effusion scores
3
2
1
0
0 2 4 6 8 24 48 72 96 120 144 168
Time (h)
D
Lameness grade
6
4
2
0
0 2 4 6 8 24 48 72 96 120 144 168
Time (h)
E
Carpal circumference (ratio)
1.10
1.05
1.00
0 2 4 6 8 24 48 72 96 120 144 168
Time (h)
F
General distress score
2.5
2.0
1.5
1.0
0.5
0.0
0 2 4 6 8 24 48 72 96 120 144 168
Time (h)

Fig. 3

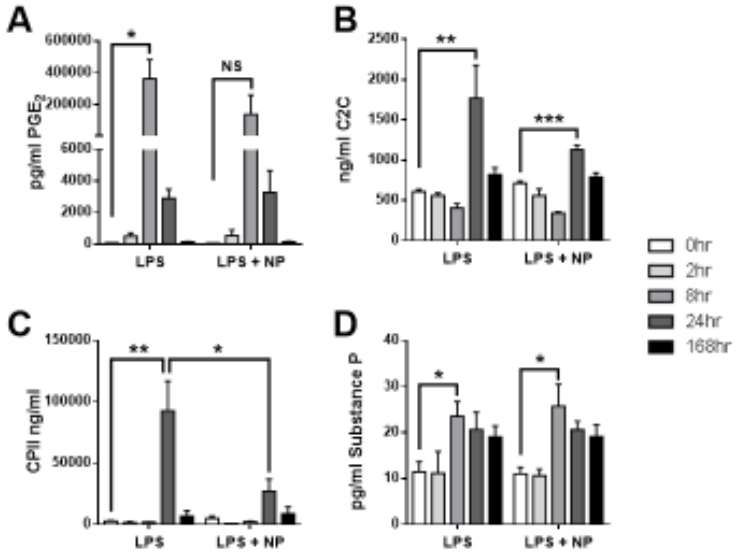
A
B
C
D
pg/ml PGE2
ng/ml C2C
CPII ng/ml
pg/ml Substance P
LPS
LPS + NP
NS
0hr
2hr
8hr
24hr
168hr

Fig. 4

Suppl. Fig. 1. Compliance of aseptic work environment. The lack of growth in settle plates, agar plates from viable air sampling, and contact plates after incubation at 25 °C (7 days) and 37 °C (7 days) validates the aseptic environment during the production process.

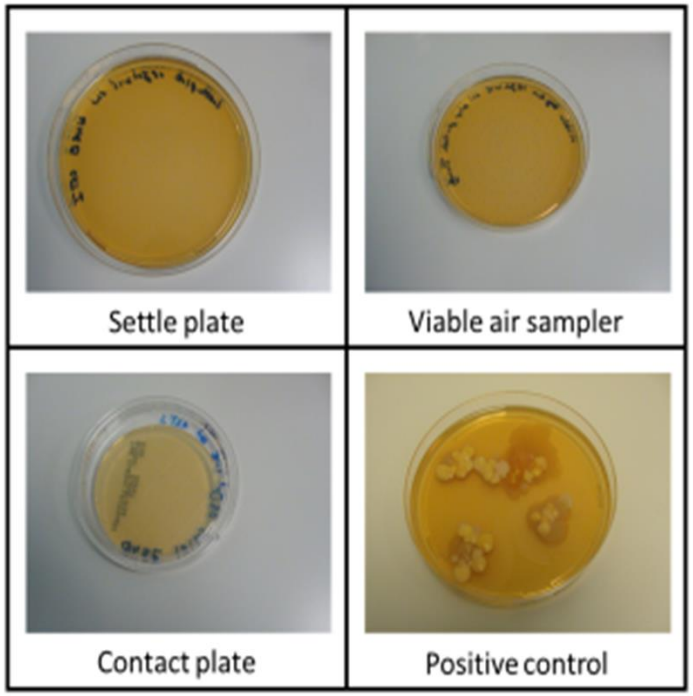

Table 1. Analytical and pharmaceutical grade materials according to product specifications. MW: molecular weight, CFU: colony forming unit, EU: endotoxin unit, ND: not determined.

	Source	**Catalogue number**	**MW**	**Total viable count**	**Endotoxin load**
HA sodium salt from *streptococcus equi sp.* (Analytical grade)	Sigma-Aldrich, Czech Republic	53747	15000 – 18000 kDa	ND	ND
Ultrapure sodium hyaluronate from *streptococcus zooepidemicus* (Pharma Grade 80)	Kibun Food Chemifa Co. LTD, Japan	4266107	620 – 1200 kDa	≤ 20 CFU/g	≤ 2.5 EU/kg
Protasan UP CL 113 CS salt (Both processes)	NovaMatrix®, Norway	4210021	< 200 kDa	≤ 100 CFU/g	≤ 100 EU/g
sCT Ph.Eur. (Both processes)	Polypeptide, Sweden	500059	3432 g/mol (free base)	≤ 100 CFU/g	< 25 EU/mg
Deionised water (Analytical grade)	(Elix® Essential Water Purification System, Merck Millipore, Ireland)	-	18 g/mol	ND	ND
Water-for-injection, free of endotoxins, ultra-filtered and autoclaved (Pharma grade)	Sigma-Aldrich, Switzerland	95289	18 g/mol	0	0

* IU/mg as stated in supplier specifications were converted to EU/mg.

Table 2. Dynamic viscosity of different grades of HA (0.1 % (w/v)) after sonication.

Min	Analytical grade (mPa*s) [a]	Pharma grade (mPa*s)
0	15.49 ± 0.82	10.45 ± 1.10*
10	8.33 ± 0.43	5.87 ± 0.20*
30	4.13 ± 0.23	3.39 ± 0.12
60	2.91 ± 0.18	2.50 ± 0.01
90	2.37 ± 0.13	2.21 ± 0.21
120	2.14 ± 0.23	1.97 ± 0.10

[a] Data from [35] with permission. Data is expressed as mean ± SD (n = 3 – 6). * P<0.001 versus analytical grade.

Table 3. Properties of NPs synthesised in low volume (12 mL) and high volume for the equine study (48 mL), using analytical and pharmaceutical grade HA (0.1 % (w/v)), as indicated.

	Particle size (nm)	PdI	ZP (mV)	Dynamic viscosity (mPa*s)
Analytical (12 mL)	163 ± 15	0.19 ± 0.04	-48 ± 6	1.27 ± 0.02
Pharma (12 mL)	187 ± 30††	0.16 ± 0.07	-30 ± 6††	0.96 ± 0.04†
Pharma (48 mL)	190 ± 17	0.17 ± 0.06	-42 ± 7**	1.14 ± 0.08*

† P<0.01 versus analytical (bench-scale); †† P<0.001 versus analytical (bench-scale); ** P<0.001 versus pharma (bench-scale); * P<0.05 versus pharma (bench-scale). Data is expressed as mean ± SD (n = 3-24).

Table 4. Association efficiency (AE) and peptide loading (PL) of NPs loaded with 100 µg/mL sCT comprising analytical (bench scale) or pharma grade HA (equine study).

	Filtrate (AE, %)	Concentrate (AE, %)	Filtrate (PL, %)	Concentrate (PL, %)
1 Analytical (bench-scale)	100 ± 1	92 ± 6	7 ± 1	7 ± 1
Pharma (equine study)	94 ± 3	93 ± 4	8 ± 1	7 ± 1

AE and PL were calculated according to concentration of sCT detected in concentrate and filtrate. Data expressed as mean ± SD (n = 4).

Table 5. Bacteriology screening of NPs used for equine studies

	Media	Batch 1	Batch 2	Batches 3-6
Aerobic culture	**Tryptone Soya Broth**	-	-	-
Anaerobic culture	**Tryptone Soya Broth**	**one sample positive for alpha-haemolytic *Streptococcus* species**	-	-
	Schaedler Anaerobic Broth	-	**one sample positive for *Staphylococcus* species**	-
Fungal culture	**Tryptone Soya Broth**	-	-	-

Concentrations: Trials refer to individual and separate batches of NPs (1 mg/ mL) comprising HA (Pharma grade), CS and sCT; Code: (-) negative for growth. Species analysed according to the European Pharmacopoeia [40].

Table 6. Endotoxin test for HA/sCT, CS and NP synthesised using either analytical or pharma grade materials

	Analytical grade (*not spiked*)	Pharma grade (*spiked*)	Pharma grade (*not spiked*)
HA/sCT	**+**	**+**	**-**
CS	**+**	**+**	**-**
NP	**+**	**+**	**-**

Concentrations: HA and CS: 1 mg/mL; sCT: 0.1 mg/mL; NP: 1 mg/mL; endotoxin: 0.4 EU/mL. Code: +, clot formed; - (no clot). Samples were spiked or not spiked with endotoxin.

Suppl. Table 1. General distress score sheet for the equine LPS model

Parameter	**Animal ID**	**Score**	**Date/Time**
Food and water intake	Normal	0	
	Moderate	1	
	Low	2	
	No food or water intake	4	
Clinical signs	Normal temperature (T), cardiac (C) and respiratory (R)rates	0	
	Slight changes	1	
	T ± 1 °C, C/R rates increase more than 30 %	2	
	T ± 2 °C, C/R rates increase more than 50 %	4	
Natural behaviour	Normal	0	
	Minor Changes	1	
	Less mobile and alert, isolated	2	
	Restless or still	4	
Provoke behaviour	Normal	0	
	Minor depression or exaggerated response	1	
	Moderate change in expected behaviour	2	
	Reacts violently, or very weak and pre-comatose	4	
	Total	**0 – 16**	

Score	**Action**
0 – 3	Normal, no action to be taken
4 – 8	Monitor carefully, consider analgesics
9 – 12	Seek second opinion from named animal care and welfare officer and/or named veterinary surgeon. Consider euthanasia.
13 – 16	Indicates severe pain. Seek immediate second opinion from named veterinary surgeon. Animal withdrawn from project. Based on advice from named veterinary surgeon, initiate appropriate treatment and analgesia. If animal's symptoms cannot be alleviated, again in consultation with the named veterinary surgeon, consider euthanasia.