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Research paper

Sustained intra-articular release of celecoxib in an equine repeated LPS synovitis model

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ABSTRACT

Synovial inflammation is an important characteristic of arthritic disorders like osteoarthritis and rheumatoid arthritis. Orally administered non-steroidal anti-inflammatory drugs (NSAIDs) such as celecoxib are among the most widely prescribed drugs to manage these debilitating diseases. Intra-articular delivery in biodegradable in situ forming hydrogels overcomes adverse systemic effects and prolongs drug retention in the joint.

In this study two formulations of celecoxib (40 mg/g and 120 mg/g) in a propyl-capped PCLA-PEG-PCLA triblock copolymer were sequentially evaluated in a multiple LPS challenge equine synovitis model. Intra-articular release and systemic exposure to celecoxib and local changes at joint level were evaluated longitudinally.

A single intra-articular injection of the high dose (HCLB)-gel or low dose (LCLB)-gel showed a sustained and controlled intra-articular release in both inflamed and healthy joints together with very low systemic exposure. Synovitis and lameness were moderate respectively very mild in this model due to the low concentration LPS (0.25 ng/joint). Both celecoxib formulations had a mild, transient effect on inflammatory and structural synovial fluid biomarkers but these returned to baseline within one week of administration. The HCLB-gel showed a significant inhibition in peak white blood cell concentration at 8 h after LPS induction.

Elevated levels of celecoxib were observed in the joint for up to 30 days but no overall anti-inflammatory effects could be observed, which was thought to be due to the moderate synovitis. As there were no long-term adverse effects, sustained intra-articular release of celecoxib from in situ forming hydrogels should be evaluated further for its effects on longer-term relief of inflammatory joint pain in humans and animals.

1. Introduction

Osteoarthritis (OA) is the most prevalent arthritic disease and a leading cause of disability in humans, horses and small animals [1–3]. While OA has historically been considered a degenerative disease of cartilage, there is now consensus that OA is a multifactorial and heterogeneous disorder of the whole joint, with a critical role for inflammation in its pathogenesis, either as a key trigger or as a secondary phenomenon [4–6]. The inflammation in OA is generally intermittent in character, often comparatively low-grade and differs from the high-grade inflammation in rheumatoid arthritis (RA) and other autoimmune diseases [2,7].

Currently available treatments for OA focus on pain relief and functional improvement, but offer no substantial disease modification. Celecoxib is the traditional first-line treatment for OA. It is a non-steroidal anti-inflammatory drug (NSAID) that selectively inhibits the pathway of cyclo-oxygenase 2 iso-enzyme (COX-2), resulting in impaired production of prostaglandins. It has been used to relieve pain and inflammation in numerous species, including humans, rats, rabbits, dogs, horses and camels [8–12]. Although celecoxib has lower gastrointestinal toxicity than less selective COX-inhibitors [13], its prolonged use and use at high doses is associated with an increased risk of thromboembolism and cardiovascular events [14,15].

Local intra-articular drug delivery would reduce drug-related systemic side effects and might be especially suitable to deliver drugs with low bioavailability [16–18]. However, rapid clearance of most active compounds means numerous injections are required for optimal treatment, causing poor patient compliance and a higher risk of complications [16,19,20]. One of the strategies to obtain sustained intra-articular drug release is encapsulation in slow-release formulations

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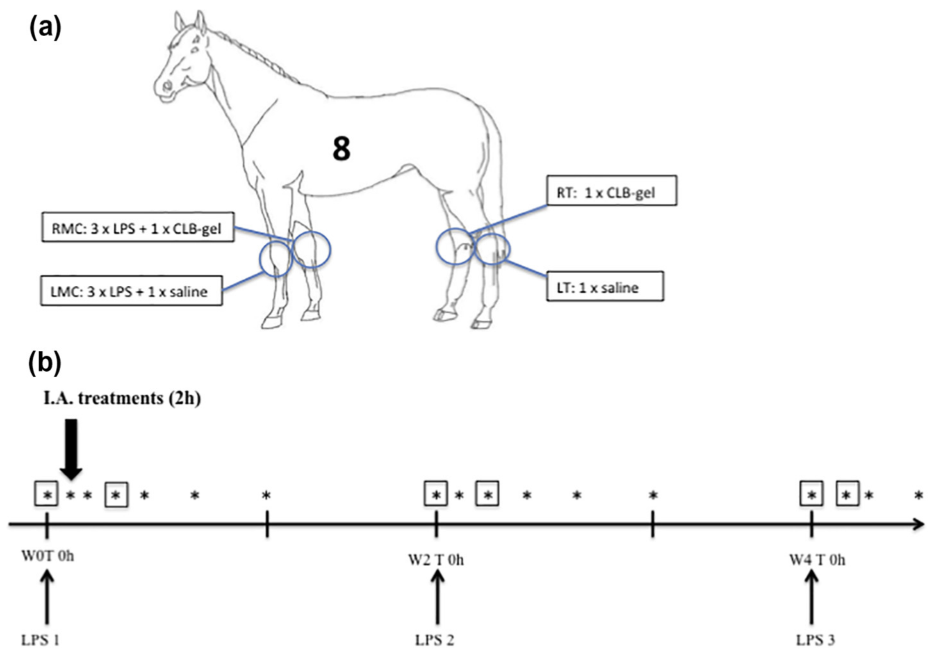

Fig. 1. Schematic view of the different intra-articular treatments in each of the 8 horses (A) and the timeline of this study (B). The time line of this *in vivo* study represents 1 period of 4 weeks and 72 h, which is repeated after a 5-week washout period: period 1: 120 mg/g CLB-gel – wash out – period 2: 40 mg/g CLB-gel. ⊡ = kinetic gait analysis (0–8 h), * = blood – synovial fluid sampling time points.

[21,22]. In particular, thermoresponsive aqueous formulations such as capped poly(ε-caprolactone-co-lactide)-b-poly(ethylene glycol)-b-poly(ε-caprolactone-co-lactide (PCLA-PEG-PCLA) triblock copolymers are suitable because of their high encapsulation efficiency, sustained and controlled release for prolonged duration and complete drug recovery [23,24]. Furthermore, they are soluble at ambient temperatures and therefore easy to inject. Once in the body, they quickly form a gel, ensuring excellent retention in the joint. These systems are suitable for loading and release of hydrophobic drugs and have recently been shown to be suitable for intra-articular delivery of celecoxib [23,25,26], but prolonged efficacy has not yet been demonstrated.

In contrast with humans and small animals, the horse is an appropriate model for frequent sampling of synovial fluid (SF), allowing the monitoring of the effect of local drug delivery by longitudinal analysis of SF biomarker profiles. The horse has also been shown to be an excellent translational animal model for OA, with treatment outcomes relevant for human OA [27–29]. In this *in vivo* study we used a well-described lipopolysaccharide LPS challenge synovitis model in horses in a sequential fashion to simulate repeated low-grade inflammatory flares. We used this model to test the long-term efficacy of treatment with a celecoxib-loaded hydrogel consisting of a propyl-capped PCLA-PEG-PCLA copolymer. In earlier studies *in vitro* release of celecoxib from this formulation was shown to start at a 10-day lag phase followed by a sustained release of 90 days, mediated by polymer dissolution from the gel [23]. Sustained release of celecoxib was also shown *in vivo* after subcutaneous injection in rats [30] and intra-articular administration was tolerated well in both rats and in horses. In the latter species, local and sustained release of celecoxib for 4 weeks with very limited systemic exposure to the drug was shown [31]. We investigated biocompatibility, release profile in SF and plasma and disease modifying effects of a single intra-articular injection of hydrogels loaded with a low (40 mg/g; LCLB) or high (120 mg/g; HCLB) dose of celecoxib. A panel of inflammation and structural synovial fluid biomarkers was used to evaluate performance of these drug delivery systems, complemented by clinical evaluations. It was hypothesized that the kinetic profiles of celecoxib in both synovial fluid and serum would mirror those observed earlier, thus confirming prolonged release of the active substance. A further hypothesis was that the joint inflammation, as induced and maintained by the repeated LPS administration, would be mitigated by the use of the gel-celecoxib combinations with the HCLB gel having a stronger effect than the LCLB gel.

2. Materials & methods

2.1. Preparation of celecoxib-loaded gels

The synthesis of the fully propyl-capped PCLA-PEG-PCLA gel was performed as previously described [31]. The gel is a mobile sol at room temperature (G″ > G′) and an immobile gel (G′ > G″) at body temperature (37 °C) after 30 min. For a full characterization of the gel including rheograms, see [32]. Celecoxib (USP Pharm.) was obtained from LC Laboratories, USA. For the system loaded with 40 mg/g celecoxib (LCLB-gel) 0.44 g celecoxib was added to 1.98 g polymer and 8.58 g phosphate buffered saline (PBS) (20 mM Na_2HPO_4, 5 mM NaH_2PO_4, 120 mM NaCl). For the system loaded with 120 mg/g celecoxib (HCLB-gel), 1.2 g celecoxib was added to 1.8 g polymer and 7 g PBS. Subsequently, the samples were autoclaved for 15 min at 120 °C. This procedure did not have significant effects on the properties of the gel as measured by gel permeation chromatography and oscillary rheology measurements [31]. After autoclaving, the samples were vortexed for ∼ 1 min, cooled down under tap water and again vortexed for ∼1 min and subsequently incubated at 4 °C for ∼48 h to yield a macroscopically homogeneous white suspension, *i.e.* a gel containing undissolved celecoxib.

These drug-loaded systems were similar to those previously described [23,31], except for the celecoxib dosages, which did not change their characteristics. Both formulations were injectable sols at 4 °C temperature and gels at 22 °C for the 'LCLB'-gel and 25 °C for the 'HCLB'-gel and could be injected through a 21G needle, which is the size mostly used for arthrocentesis in the horse.

2.2. Animal model

Eight Dutch Warmblood horses (age mean ± s.d.: 6.0 ± 0.9 years, weight: 501 ± 69 kg, sex: 6 female - 2 castrated male horses) were used, which were all clinically sound at the walk and at trot and had no joint effusion or radiographic abnormalities of the involved joints. Horses were housed individually in 3.0 × 3.0 m stables throughout the study. A 2-period placebo-controlled design was adopted, in which left and right middle carpal (LMC and RMC) and talocrural joints (LT and RT) and order of treatments (placebo vs. 'CLB-gel') were predetermined for each animal (Fig. 1A), with a 5-week washout period between the first ('HCLB', 120 mg/g) and second ('LCLB', 40 mg/g) experimental periods.

The LPS aseptic synovitis model as described by De Grauw et al. [33] was used, adapted for sequential challenging. Sterile LPS from Escherichia Coli 055:B5 (catalogue number L5418-2ML, Sigma Aldrich Chemie B.V., The Netherlands) was diluted to a final concentration of 0.25 ng/ml in phosphate buffered saline solution (PBS). On day 0 (T0), both middle carpal joints were injected with 0.25 ng LPS (LPS1). After two hours (T2) the right middle carpal joint and the right talocrural joint were treated with a single intra-articular injection of 2 ml of celecoxib-loaded gel and the left middle carpal joint and left talocrural joints were treated with 2 ml saline (placebo). Subsequently, repeated induction of synovitis was effectuated by injection of 0.25 ng LPS (LPS 2 and LPS3) in week 2 and 4 of each period in the middle carpal joints (Fig. 1B). The study ended at T72 after LPS 3 (total duration 4 weeks and 72 h).

The study design was approved by the institutional Ethics Committee on the Care and Use of Experimental Animals in compliance with Dutch legislation on animal experimentation.

2.3. Evaluation of clinical response

Horses were monitored throughout the study for signs of discomfort. Temperature, pulse, and respiration at rest were measured daily.

For objective lameness evaluation, a floor mounted force plate (Kistler Z4852C, Kistler Instrument Corp, USA) was used to determine peak vertical force, vertical impulse and stance duration. Force plate assessments were performed every 2 weeks at baseline and at T8 after each LPS injection. At each time point, 3–5 valid observations were recorded for each forelimb and adjusted for body weight. Calibration of the force plate was verified prior to and repeated shortly after the study.

2.4. Collection of synovial fluid and serum

Synovial fluid samples (~2 ml) from the middle carpal joints (left: LPS + saline, right: LPS + CLB-gel) were aspirated at time points T 0, 4, 8, 24, 72 and 168 h after each LPS injection, during both periods of 4 weeks and 72 h. Synovial fluid samples of control talocrural joints (left: saline, right: CLB-gel) were aspirated at time points (W0) T 0, 4, 8, 24, 72 and 168 h, and then weekly at T0, during both periods. A portion of the synovial fluid was placed in EDTA tubes for white blood cell count (WBC) and total protein (TP) measurement. The remainder was centrifuged in plain tubes at 3000*g* for 10 min, aliquoted and stored at −80 °C. Blood was collected following the same scheme from the left jugular vein in heparinized vials and spun down for 5 min at 1500*g* to produce plasma.

2.5. Synovial fluid analysis

The celecoxib and prostaglandin E2 (PGE_2) concentrations were determined by high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS) analysis as described previously [33–35]. For extraction from synovial fluid or serum, internal standard (celecoxib-tolyl-d7 or 16,16-dimethyl $PGF_2\alpha$) was added to 100 µl sample. Then, 0.1 M sodium acetate buffer (pH 5) and ethyl acetate were added and the samples were mixed thoroughly, centrifuged at 8000*g* for 10 min and stored at −20 °C overnight. The upper ethyl acetate phase was transferred into a new tube. This ethyl acetate extraction was repeated once, and after evaporation of the pooled solvents; the residue was dissolved in methanol and measured. Data were analyzed with Analyst software version 1.4.2 (Applied Biosystems, The Netherlands). Concentrations were calculated from peak areas relative to the internal standard and standard curve.

Synovial fluid WBC and total protein concentrations were determined using a flow cytometer (Advia 210i System - Siemens) and refractometer [32], respectively. Synovial fluid samples were evaluated for glycosaminoglycan (GAG) concentrations using a modified 1,9-dimethylmethyleneblue assay adapted for use in microtitre plates [34]. Concentrations of C2C, a neo-epitope present on collagenase-cleavage fragments of type II collagen, and CPII, carboxy-terminal propeptide of type II collagen, were measured by use of commercial ELISA kits (IBEX Technologies, Quebec, Canada) in accordance with the manufacturer's recommendations. General matrix metalloproteinase (MMP) activity was measured using the fluorogenic substrate FS-6 (Calbiochem, San Diego, CA, USA). In short, samples were diluted 20-fold in MMP buffer (0.1 M Tris, 0.1 M NaCl, 10 mM $CaCl_2$, 0.05% [w/v] Triton X-100, 0.1% [w/v] PEG-6000 and 5 µM FS-6, pH 7.5). Samples were added in triplicate to a black 384-well microplate and the fluorescent signal was monitored continuously for 45 min at 37 °C using a clariostar microplate reader. The slope of the resultant linear curve (relative fluorescence units/s [RFU/s]) was calculated as a measure of general MMP activity and corrected for the negative control (5 mM EDTA).

2.6. Statistical analysis

Data are presented as mean ± SEM and were compared using two-way repeated-measures ANOVA with Tukey's (to compare effects over time) or Dunn-Sidak (to compare treatments) post-hoc tests. Normality was tested using the Shapiro-Wilk test. Statistical analyses were performed using GraphPad Prism 7 for Mac. The level of significance was set at $p < 0.05$.

3. Results

3.1. Clinical evaluation and quantitative gait analysis

In all 8 horses, there were no abnormalities in general behavior, appetite, heart rate, respiratory rate and rectal temperature throughout the experiment. All horses were sound at the start of each treatment period.

The peak vertical force decreased significantly at T8 after LPS1, LPS2 and LPS3 (all $p < 0.01$, compared to baseline) in the 'HCLB' period, in the saline treated left leg. The same trend was observed for the celecoxib treated right leg but this did not reach significance. In contrast, in the 'LCLB' period, there was a significant decrease in peak vertical force observed in the right (celecoxib-treated) leg (T8 after LPS1 $p = 0.0001$, T8 after LPS2 $p = 0.03$), but significance was not reached in the left (saline) leg. No significant differences were observed between treatment groups in either period.

The vertical impulse increased in the celecoxib-treated right leg in the 'HCLB' period (T8 after LPS1 and LPS2 both $p < 0.01$). No significant differences were observed for the saline treated left leg in the HCLB period. However, the vertical impulse was significantly lower in the saline treated leg at T8 after LPS2 ($p = 0.01$ compared to the celecoxib-treated leg). In the 'LCLB' period the vertical impulse was increased in both legs only after LPS3 ($p < 0.05$, compared to baseline) and there were no significant differences between treatments.

Stance duration increased significantly at T8 after LPS1 in the 'HCLB' period in both front legs ($p < 0.01$), and was higher at T0 and T8 after LPS3 ($p < 0.05$). In the 'LCLB' period stance duration was only significantly elevated at T8 after LPS3 ($p < 0.05$). There were no significant differences in stance duration between treatments in either period.

3.2. Synovial fluid analysis

3.2.1. In vivo release of celecoxib from gels administered intra-articularly

The celecoxib concentration in synovial fluid showed a different time curve for each dose and for the carpal/talocrural joints (Fig. 2A). For both 'HCLB' and 'LCLB' formulations, the C_{max} of celecoxib in synovial fluid was reached after 6 h (T8 after LPS1). For the 'HCLB'-gel C_{max} was 25.6 ± 11.7 µg/ml in the LPS-induced right middle carpal joint vs. 42.9 ± 23.8 µg/ml for the right talocrural control joint. For the 'LCLB'–gel C_{max} was 16.6 ± 5.6 µg/ml in the right talocrural control joint (the right middle carpal joint was not measured). After

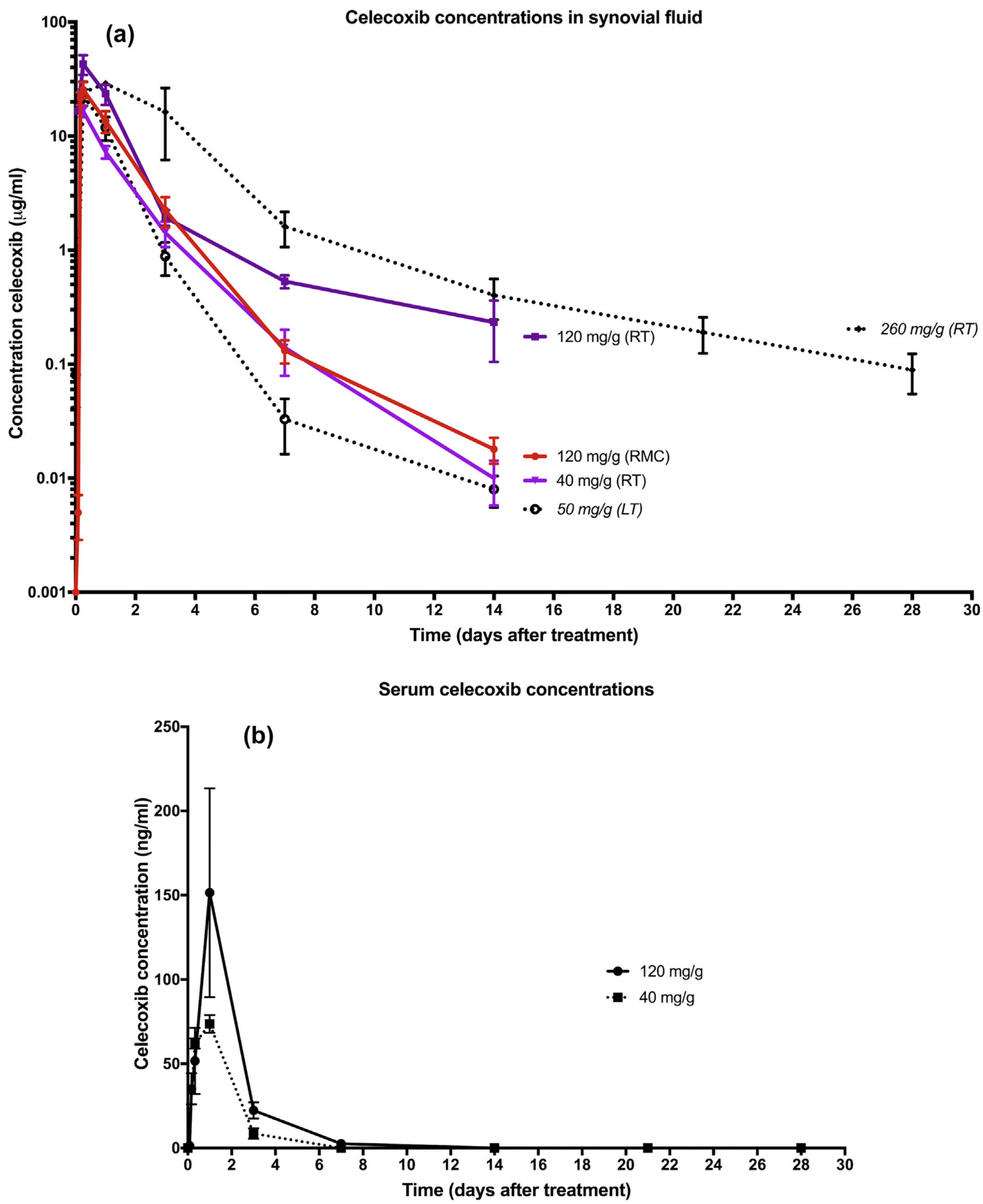

Fig. 2. Celecoxib concentrations in (A) synovial fluid and (B) serum after intra-articular administration of 'HCLB gel' and 'LCLB gel' loaded with 120 and 40 mg celecoxib per g gel, in the right middle carpal (RMC) and right talocrural (RT) joint respectively. Data for the 40 mg dose in the right middle carpal joint were not available for technical reasons. Dotted lines indicate concentrations observed in the study by Petit et al. [31]. Note the differences in scaling of the Y-axes.

reaching C_{max}, the celecoxib concentration in the joints treated with the 'LCLB'-gel decreased more rapidly, reaching a concentration of 0.14 ± 0.17 µg/ml 7 days post treatment and dropped below 0.01 µg/ml 14 days post treatment. The celecoxib concentration in the joints treated with 'HCLB'-gel was 0.53 ± 0.20 µg/ml (right talocrural joint) and 0.13 ± 0.09 µg/ml (right middle carpal joint) at day 7 and below 0.2 µg/ml (right talocrural joint) vs 0.01 µg/ml (right middle carpal joint) at day 14. The total areas under the curve (AUC) were 63.7 ± 17.18 µg·h/ml ('HCLB', right talocrural joint), 38.78 ± 10.79 µg h/ml (HCLB, right middle carpal joint) and 23.36 ± 4.18 µg h/ml ('LCLB', right talocrural joint), which is an approximately factor 3 difference in the right talocrural joint in which both doses were injected.

The C_{max} of celecoxib in plasma was reached at 22 h (Fig. 2B) and was 0.15 ± 0.17 µg/ml for the 'HCLB'-gel and 0.073 ± 0.013 µg/ml for the 'LCLB'-gel. The AUC in plasma was 0.31 ± 0.399 µg h/ml for the 'HCLB'-gel and 0.155 ± 0.022 µg/ml for the 'LCLB'-gel. The plasma C_{max} and AUC values were thus both approximately 150 times lower than in synovial fluid for the 'HCLB'-gel.

3.2.2. White blood cell count

In both LPS-stimulated carpal joints, there was a significant but transient rise in SF WBC count with a peak at T8 after LPS1 ($p < 0.0001$) during both treatment periods (Fig. 3A, B). After LPS2 this increase was much smaller and did not reach significance. After LPS3 there was only a significant peak in the 'HCLB'-gel treated right middle carpal joints (T8 $p = 0.0025$ compared to baseline). The WBC count was significantly lower in the 'HCLB'-gel treated right middle

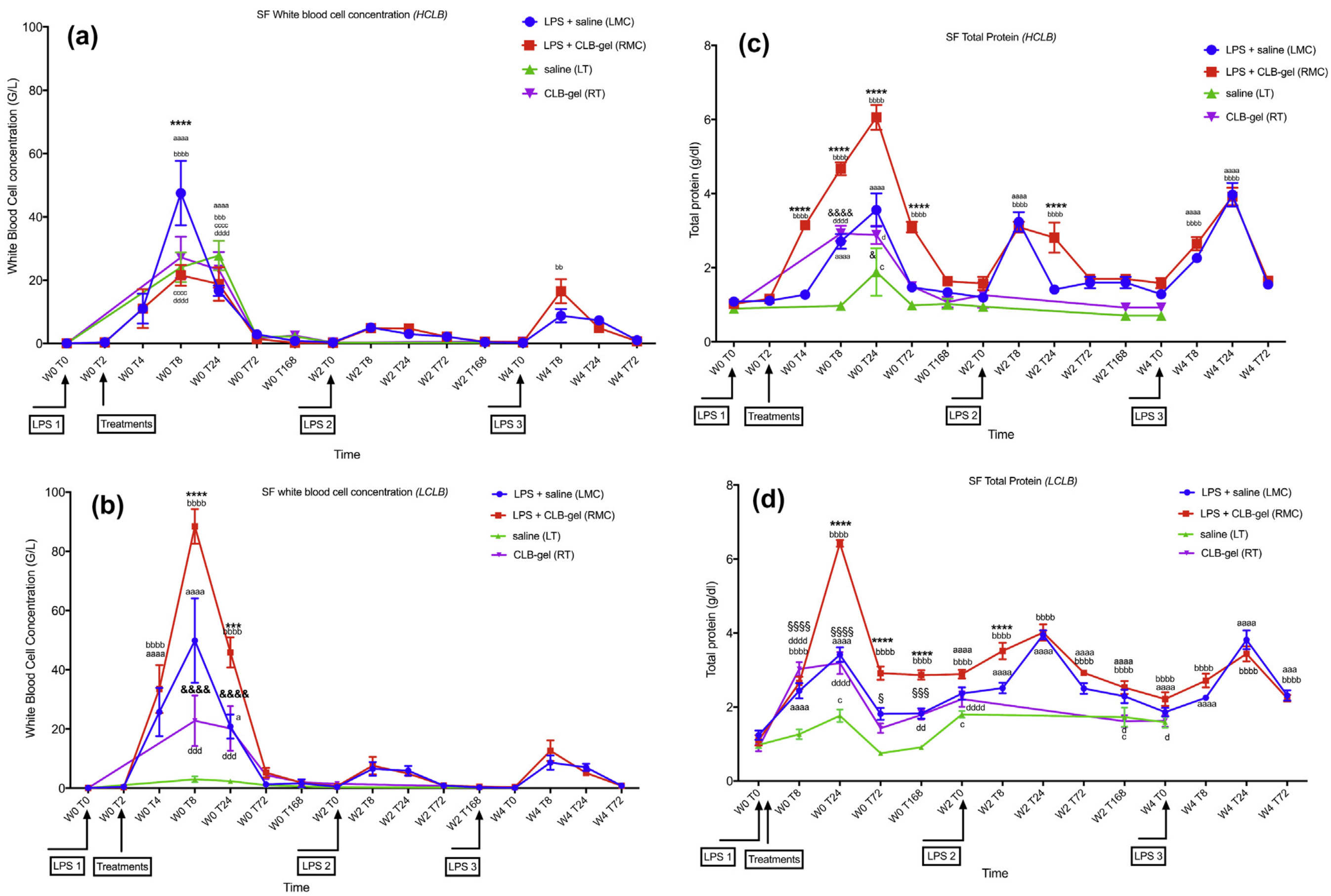

Fig. 3. Mean ± SEM synovial fluid (SF) concentrations of white blood cells (A, B) and total protein (C, D) measured during the 'HCLB gel' (120 mg/g) period (left) and the 'LCBL gel' (40 mg/g) period (right). Significant differences between treatment groups in LPS-treated middle carpal joints and talocrural control joints, are indicated with the symbols (*) and (&) respectively. Letters are used to indicate the significant differences compared to baseline in the left (a) and right (b) middle carpal joints and left (c) and right (d) talocrural joints. One symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$ and four symbols: $p < 0.0001$.

carpal joints at T8 after LPS1 ($p < 0.0001$) compared to saline treated LMC joints (Fig. 3A). However, the situation was reversed during the 'LCLB' period (Fig. 3B) with a higher WBC count in the 'LCLB'-gel treated joints (both RMC and RT) at T8 and T24 after LPS1 (both $p < 0.0001$). In the talocrural control joints, a peak in SF WBC count was also observed in both joints at T8 and T24 ($p < 0.0001$) in the 'HCLB' treatment period. In the 'LCLB' period this peak only occurred in the gel-treated joint.

3.2.3. Total protein

In both LPS-stimulated carpal joints, there was a significant rise in SF total protein with a peak at T8-T24 after each LPS challenge during both treatment periods (all $p < 0.0001$ compared to baseline) (Fig. 3C and D). Significantly higher SF total protein was observed in both celecoxib treated groups compared to saline after LPS 1 ($p < 0.0001$). In the talocrural control joints, a relatively small increase in SF total protein with a peak at T24 was observed in both joints in the 'HCLB' period (LT $p < 0.05$, RT $p < 0.0001$) and in the 'LCLB' period (T24 LT $p < 0.01$, RT $p < 0.0001$). Significantly higher peaks were observed in the celecoxib treated talocrural joints compared to saline ('HCLB' T8, T24 $p < 0.0001$ and 'LCLB' T8, T24 and T168 $p < 0.0001$, T72 $p < 0.05$). During the 'LCLB' period total protein remained slightly, but significantly elevated in the talocrural joints at later time points without significant differences between treatment groups.

3.2.4. Prostaglandin E_2

Significantly elevated prostaglandin E2 (PGE_2) concentrations were observed in the saline treated left middle carpal joint at T8 after LPS1 ($p < 0.005$), at which moment the PGE_2 concentration was also significantly higher than in the 'HCLB'-gel treated contralateral RMC joint (Fig. 4A). No other significant differences were observed in either period for the carpal or talocrural joints (Fig. 4A and B).

3.2.5. General matrix metallo proteinase activity

General matrix metalloproteinase (MMP) activity peaked significantly in the 'HCLB'-gel treated right middle carpal joints at T24 after LPS 1 ($p < 0.0001$ compared to baseline and $p < 0.005$ compared to left middle carpal joints) and at T8 after LPS2 ($p = 0.02$ compared to baseline) (Fig. 4C). During the 'LCLB' period (Fig. 4D), MMP activity was overall lower, but was significantly increased in the 'LCLB'-gel treated joint at T4 after LPS1 ($p = 0.004$ compared to baseline and $p < 0.001$ compared to the saline-treated left middle carpal joints). At T24 the situation had reversed with a significantly lower level in the 'LCLB'-gel treated joint ($p = 0.02$) compared to saline treated joints. In the talocrural control joints, general MMP activity compared to baseline showed significant peaks at T8 and T24 after LPS1 in 'LCLB'-gel treated joints ($p = 0.02$ and $p < 0.001$, respectively) (Fig. 4D), which differed significantly from the saline-treated contralateral joint at these points in time.

3.2.6. Glycosaminoglycan release

In both treatment periods there was a significant peak in glycosaminoglycan levels at T24 after LPS1 in all LPS-stimulated joints (all $p < 0.0005$) (Fig. 5A and B). After LPS2 there was a significant peak at T24 in the 'HCLB'-gel and 'LCLB'-gel treated joints (both $p < 0.05$) and in the saline treated joint during the 'LCLB' period ($p < 0.01$). Finally,

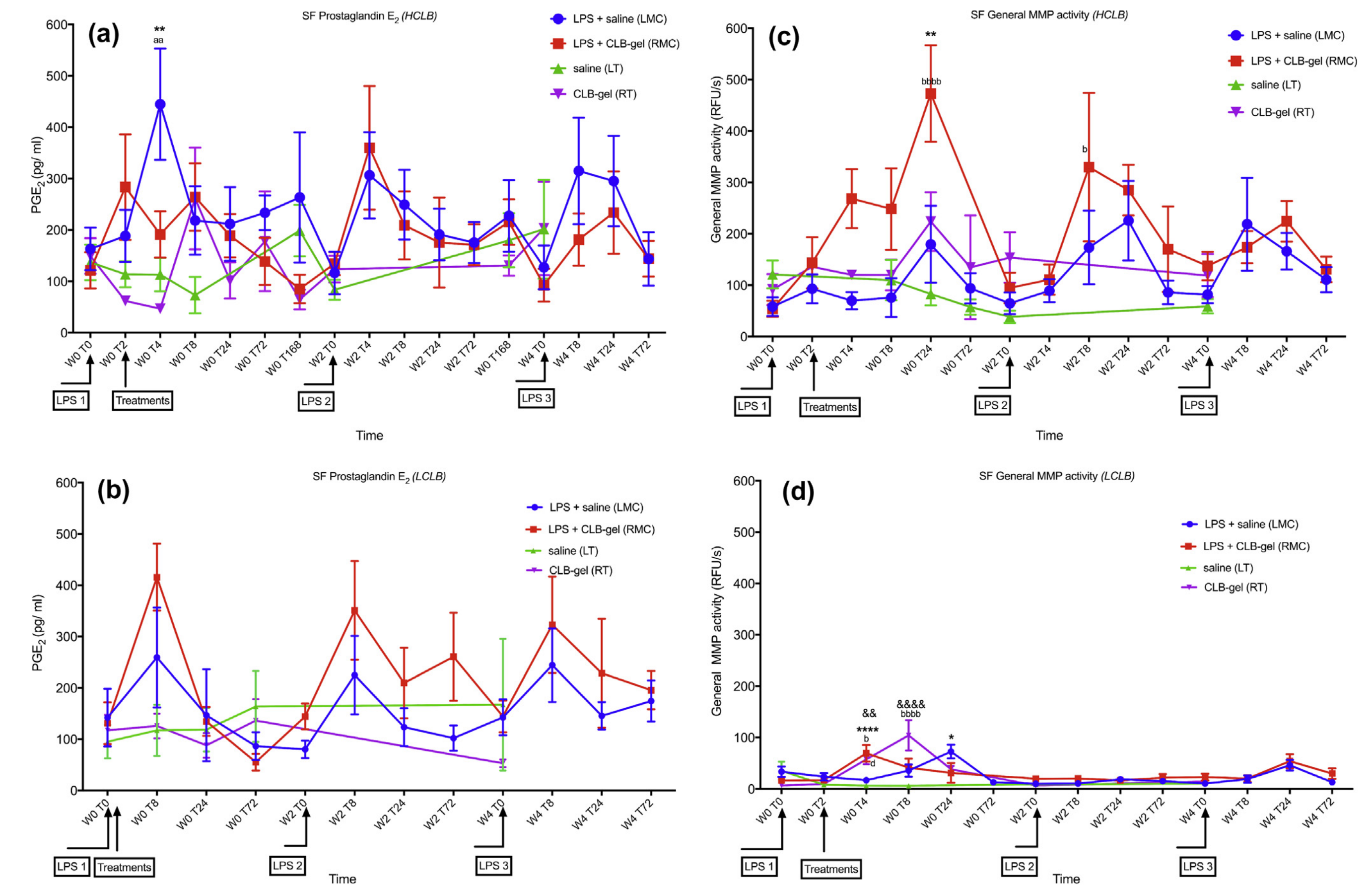

Fig. 4. Mean ± SEM synovial fluid (SF) concentrations of prostaglandin E_2 (A, B) and MMP activity (C, D) measured during the 'HCLB gel' (120 mg/g) period (left) and the 'LCBL gel' (40 mg/g) period (right). Significant differences between treatment groups in LPS-treated middle carpal joints and talocrural control joints are indicated with the symbols (*) and (&) respectively. Letters are used to indicate the significant differences compared to baseline in the left (a) and right (b) middle carpal joints and left (c) and right (d) talocrural joints. One symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$ and four symbols: $p < 0.0001$.

at T24 after LPS3 there was a significantly higher peak in the 'LCLB' treated joint compared to baseline ($p < 0.01$). The peak after LPS1 was a significantly higher in both 'HCLB'-gel and 'LCLB'-gel treated joints compared to controls (both $p < 0.0001$). In the talocrural control joints SF GAG concentration showed a significant peak at T24–T72 after LPS 1 in both 'HCLB'-gel (T24 $p < 0.0001$) and 'LCLB'-gel (T24 $p = 0.05$ and T72 $p < 0.0001$) treated joints compared to baseline. The peak in the 'LCLB' treated joint was significantly higher compared to saline (T72 $p = 0.001$). In the saline treated control joints there was only a small significant peak in the 'HCLB' period (T24 $p = 0.03$).

3.2.7. Collagen type II cleavage marker (C2C)

During the 'HCLB' period the peak in C2C concentration observed at T24 - T72 h after LPS-stimulation only reached significance after LPS2 (both limbs $p < 0.0001$) but there were no significant differences between treatments (Fig. 5C and D). During the 'LCLB' period, there were significant peaks after LPS1, LPS2 and LPS3 in the saline treated left middle carpal joints (T24 all $p < 0.05$) and only after LPS2 and LPS3 in the 'LCLB'-gel treated joints (T24 $p < 0.01$ and T72 $p < 0.005$ respectively). At T24 after LPS 1, C2C levels were significantly higher in the saline treated left middle carpal joints compared to the 'LCLB'-gel treated right middle carpal joints ($p = 0.04$).

In the talocrural control joints the only significant peak was in the 'LCLB'-gel treated joints at T72 after LPS2 ($p = 0.02$).

3.2.8. Carboxy propeptide of collagen II (CPII)

There was a significant peak in CPII concentrations at T72 after LPS1 in the 'HCLB'-gel treated right middle carpal joint but not in the saline treated left middle carpal joint ($p < 0.0001$ compared to baseline and saline) (Fig. 5E). After LPS2 there was a significant peak in both 'HCLB'-gel and saline groups (both $p < 0.0001$) that was also significantly higher in the 'HCLB'-gel treated right middle carpal joints compared to saline treated LMC joints (T72 $p < 0.0001$). During the 'LCLB' period, there were significant peaks after LPS1 and LPS2 in the 'LCLB'-gel treated right middle carpal joints (T72 $p < 0.0001$ and T24 $p < 0.001$ respectively) but in the left middle carpal control joints concentrations peaked only at T24 after LPS1 ($p = 0.001$) (Fig. 5F). In the talocrural control joints there were no significant effects in the 'HCLB' period, but there was a significantly higher SF CPII concentration in 'LCLB'-gel treated right talocrural joints at T72 after LPS 1 ($p = 0.0002$ compared to baseline and saline).

4. Discussion

Sustained local delivery of NSAIDs encapsulated in biomaterials is seen as a promising approach towards long-term relief of synovial inflammation and associated joint pain in humans and animals [18,20]. Following up on the promising results of an earlier safety and kinetics study with a PCLA-PEG-PCLA copolymer loaded with celecoxib for intra-articular delivery in the horse [31], the current study sought to prove clinical efficacy of this sustained delivery system. To this end, the system was used in a repeated intra-articular LPS model, meant to provoke persistent low-grade inflammation over a period of at least 5 weeks. Outcome parameters were both functional (quantitative gait analysis) and biological (an array of validated biomarkers representing both the inflammatory and the metabolic status of the joint).

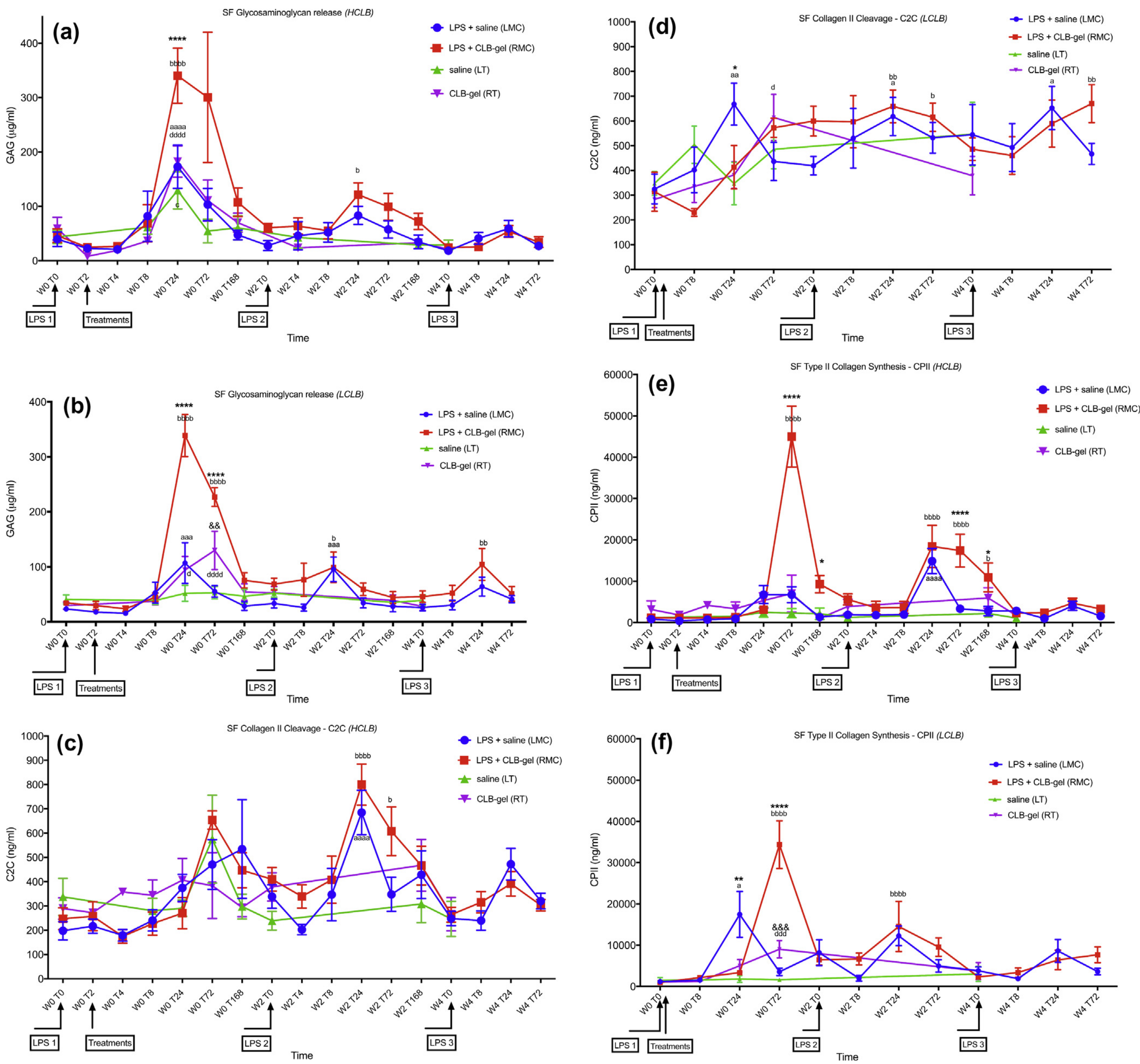

Fig. 5. Mean ± SEM synovial fluid (SF) concentrations of glycosaminoglycans (A, B), collagen II cleavage fragments, C2C (C, D) and the synthesis marker carboxypropeptide of type II collagen, CPII (E, F) measured during the 'HCLB gel' (120 mg/g) period (left) and the 'LCBL gel' (40 mg/g) period (right). Significant differences between treatment groups in LPS-treated middle carpal joints and talocrural control joints are indicated with the symbols (*) and (&) respectively. Letters are used to indicate the significant differences compared to baseline in the left (a) and right (b) middle carpal joints and left (c) and right (d) talocrural joints. One symbol: $p < 0.05$, two symbols: $p < 0.01$, three symbols: $p < 0.001$ and four symbols: $p < 0.0001$.

The safety and kinetic outcome of the previous study was almost identical to that of the current study, confirming our first hypothesis. No adverse effects were seen, either locally or systemically. The low dose ('LCLB') with which the gel was loaded in the current study was marginally lower than in the former study (40 mg/g versus 50 mg/g), but the higher dose ('HCLB') was more than halved (120 mg/g versus 260 mg/g). This was done because in the earlier study celecoxib was shown to form a paste which was very difficult to inject, due to increased presence crystals at the higher dose (260 mg/g), while being fully dissolved at the lower one (50 mg/g). The increased presence of these crystals was thought to be implicated in the transient, but clinically evident lameness that was seen in 4 of 5 horses after injection of the high celecoxib gel, but not of the low celecoxib gel [31]. Notwithstanding the differences in loading dose, the celecoxib concentrations in synovial fluid in the non-LPS challenged joints and in serum were almost identical to those earlier measured with the higher concentrations of celecoxib (Fig. 2A and B), confirming a similar release pattern. The C_{max} value after the high dose in the talocrural joint after two weeks was 42.9 μg/ml at T8 and was still 0.2 μg/ml after two weeks. Little is known about the effective concentration of celecoxib in equine joints, but these figures compare favorably with the values found in a human study [36] in which in only six out of seventeen patients celecoxib could be detected in synovial fluid (against in 17/17 in serum) at levels of 0.33–0.79 μg/ml after oral treatment at a dose of 200 mg/kg, which is a commonly used dosage in man and is thought to be effective in reducing joint pain and improving physical function [37]. Plasma concentrations in that study ranged from 0.35 to 1.85 μg/ml; in the current study the plasma concentration was 150-fold less than the intra-articular concentration and can hence be considered to be negligible in terms of potential provocation of side-effects. In this context it should be realized that in the current study design two different joints were injected at time point two hours after induction of the transient synovitis with LPS. This implies that the systemic levels measured in the two legs of the study (Fig. 2) say something about intra-articular application of twice the high (120 mg/g) and low (40 mg/g) doses, not of single doses. Plasma C_{max} values occurred also at a later time point (24 h) in plasma than in SF (6h). As the capped PCLA-PEG-PCLA thermogels swell in SF and degrade by erosion, the release kinetics likely follow different kinetics than first order [16,23,31]. Although the kinetic patterns of celecoxib were virtually identical to those in the earlier study in the non-LPS challenged joints, it should be noted that in the LPS challenged joint treated with the high (120 mg/g) dose of celecoxib release was quicker and resembled that of the low doses in both studies (Fig. 2).

The intra-articular injection of bacterial lipopolysaccharide is a well-known model for the induction of acute and transient arthritis, which has been well characterized in horses [32,38]. The model was first described by Firth et al. [39], but more thoroughly explored by Palmer and Bertone [40], who performed a titration study. Using a wide dosage range from 5000 to 0.125 ng/joint, they concluded that a dose of 0.5 ng did not lead to systemic side effects and had a maximal effect on routinely measured SF parameters. The model has been used frequently since, mostly with the originally advised dose of 0.5 ng/joint [27,32,41–43]. Repeated application of LPS to sustain a prolonged inflammation has been used much less often, but was already explored by Palmer and Bertone in their 1994 landmark study. In that study, repeated injection of 0.125 ng LPS succeeded in maintaining an inflammatory state during a week. In another study, Kay et al. [44] used a much higher dose of 100 ng/joint in an experimental design in which horses were injected intra-articularly at days 5, 10 and 15 and then after a 7-day wash-out period again at 5-day intervals for the assessment of a variety of treatments. In that study, lameness scores reached a maximum of 3.7 on a 0–5 scale, which was indicative of a severe lameness. In the current study we aimed at creating a mild lameness both for ethical reasons and because a bilateral lameness was induced by the simultaneous injection of both middle carpal joints, limiting the capability of the animal to unload the affected limb by shifting weight to the contralateral limb. For this reason, a relatively mild dose of 0.25 ng was chosen, which was lower than the most commonly used dose of 0.5 ng, but more than the 0.125 ng that had been shown to result in sustained inflammation during a week.

The lameness that was effectuated in the current study turned out to be very mild. When lame, horses use to cope with this condition by unloading the affected limb, *i.e.* reducing peak vertical force by four compensatory mechanisms. These are: reduction of total vertical impulse per stride; selectively decreasing the diagonal impulse in the lame diagonal; shifting of the impulse within the lame diagonal to the hind limb and in the sound diagonal to the forelimb; and reducing the rate of loading of peak forces by prolonging stance duration [45]. After induction, the kinetic measurements in this study indeed showed a decrease in peak vertical force and increase in stance duration, indicative of the lameness. The effects were not large in general and the effect on vertical impulse was more ambivalent, but it should be realized that in this study LPS was injected bilaterally, impeding the contralateral weight-shift that is the hallmark of unilateral lameness. Peak vertical force remained lower than baseline during both treatment periods and did not show an intermittent pattern following the repeated LPS injections, as perhaps might have been expected. Whereas a reaction to the first challenge with LPS (LPS1) was seen in all synovial fluid parameters that are classic clinical indicators of joint inflammation, an intermittent response to the repeated LPS challenge was not observed in all of these parameters. White blood cell count showed large peaks after LPS1 in both the 'HCLB' and the 'LCLB' periods, but the peaks after LPS2 and LPS3 were much smaller and did not reach statistical significance. In contrast, total protein peaks showed a typical intermittent pattern with clear reactions to all LPS stimulations. Although the graphs for PGE_2 visually showed three peaks, rises in concentration after LPS2 and LPS3 were not significant, due to the high variation. Glycosaminoglycans on the other hand showed peaks after LPS1 and LPS2 in the 'HCLB' group and after all three challenges in the 'LCLB' group. Overall, it can be stated that the LPS model worked fine after LPS1 and had some effects after LPS2 and LPS3, but those effects were less consistent and much less marked than after LPS1. LPS tolerance in response to repeated endotoxin exposure every 2 weeks might be responsible for these attenuating immunologic effects [46].

In the earlier study [31] the experimental design was different to the one used in this study. In that study a commercial hyaluronic acid product, known to produce a transient inflammatory flare, was used as a positive control and a condition with empty gel was used as the "placebo" treatment in order to assess the effect of the addition of the active celecoxib to the carrier. In the current study, there was no positive control, but an injection with saline was used as a "placebo" control, and the two loaded gels were the experimental conditions. Neither the original safety and kinetics study [31] nor the current study contained a condition with celecoxib only. This was deemed unnecessary as synovial fluid is an ultrafiltrate of blood plasma with free exchange of all molecules with a molecular weight lower than 10KDa [47], which is about 30-fold the molecular weight of celecoxib. Data on white blood cell count, total protein and glycosaminoglycan release in the earlier study showed clear rises after injection of the positive control and the high-dose celecoxib-gel (attributed to paste formation as alluded to above), but a rise was also observed, compared to baseline, after injection of the gel alone. This rise was most outspoken in the case of total protein and was annihilated by the low-dose celecoxib-gel combination, suggesting some therapeutic efficacy. In the current study there were also rises in white blood cell count, total protein and glycosaminoglycans in the talocrural control joints after injection of the celecoxib-gel in week 0. The saline injections resulted in peaks as well, but these were significantly lower than with the celecoxib-gel in all cases except for white blood cell count and glycosaminoglycans in the 'HCLB' condition. Interestingly, significantly higher levels were observed in the combination LPS plus gel, compared to LPS plus saline for all markers except for white blood cell count and PGE_2 in the 'HCLB' condition. This was also true for general MMP activity in both 'HCLB' and 'LCLB' conditions. MMP activity can also be seen as an inflammatory marker, but was not measured in the earlier study. These transient inflammatory effects were unexpected findings that disproved our second hypothesis, that the celecoxib-gel would mitigate the effects of LPS.

There are several factors that may play a role and might alone or in conjunction explain these observations. It is of course also possible that the formulation of celecoxib as used in this study, which was slightly different in terms of doses to the one in the preceding study [31] did not have enough efficacy. However, it is thought unlikely that the celecoxib dose, which was lower than in the earlier study in the 'HCLB' condition, has played a major role here, as the cases in which there seemed to be a mitigation of the LPS effect compared to control (white blood cell count and PGE_2 after LPS1) were all seen in the 'HCLB' condition. It seems more likely that in this study there was an inflammatory effect of the bare gel, that could only in certain cases be counteracted by the high celecoxib-dose (but not by the low one). A comparable effect has been reported after intra-articular injection with celecoxib-loaded PEA microspheres in a knee OA model in sheep [48]. The fact that in our former study [31] the low dose celecoxib apparently was able to counteract such effect, and in the current study only the higher dose, may suggest that the inflammatory effect of the gel was more pronounced in this study than in the former. The major difference with the former study was of course the LPS-induced arthritis and it may well be that some potentiation or other direct or indirect interaction occurs between the celecoxib and/or gel and the LPS, or that the gel behaves differently in an inflamed environment than in a naïve one. Such an interaction can also explain the lower celecoxib concentration in the

'HCLB' condition of the LPS challenged joint middle carpal joint compared to the same condition in the non-challenged talocrural joint, as depicted in Fig. 2.

The apparent pro-inflammatory effect of the gel that may have been potentiated by the presence of (LPS-induced) synovitis complicates the evaluation of the effects that were observed on the metabolic markers in this study, but the intensifying effect of the gel-CLB on the LPS model seems to be present in most metabolic markers as well. This is true for the GAG and MMP data, as said, that can to a certain extent also be seen as reflective of the inflammatory status of the joint, but also for the collagen anabolic marker CPII. LPS induces an increase in CPII 24 h after induction that lingers on for a prolonged period [33,34,38], which was also seen in the current study after LPS1 and LPS2 in both 'HCLB' and 'LCLB' conditions. Compared to the saline treated controls, this effect was significantly larger in the CLB–gel treated joints at 72 h post-LPS, which could be explained by the known chondroreparative effects of celecoxib after systemic and intra-articular administration that have been reported in humans, rats and rabbits [49–52].

The catabolic marker C2C was also previously shown to induce peak at 24 h after induction with 0.5 ng LPS [33,34,38] but not after 0.25 ng [38]. In the current study, some peaks were seen at 24 h compared to baseline ('HCLB' after LPS2 and "LCLB' after all inductions). There are no other literature data on C2C levels after a 0.25 ng LPS challenge, but it is not improbable that this catabolic marker also peaks after the lower LPS dose; the difference with the study of Lucia et al. [38] may be explained by the fact that they used immature horses that can be supposed to be in an anabolic rather than a steady-state mode and hence will be less susceptible to a catabolic LPS effect. The only difference between treatments in the current study was a lower C2C level at 24 h after LPS 1 in the 'LCLB' condition, so there does not seem to be an indication of any aggravating effect of the gel in relation to this marker.

5. Conclusion

It can be concluded that the kinetic profiles of celecoxib in this study wholly agreed with those seen in an earlier safety and kinetics study, confirming the potential of the gel-NSAID combination that was tested for prolonged release of the active substance. The high dose and low dose celecoxib formulations had a mild, transient effect on inflammatory and structural synovial fluid biomarkers, but these returned to baseline within one week of administration. Elevated levels of celecoxib were observed in the joint for up to 30 days, but no overall anti-inflammatory effects could be observed, which was thought to be due to the moderate synovitis. The tested gel-active compound can be considered potentially promising, nevertheless this study underlines the necessity of extensive and exhaustive testing of novel controlled release based medication systems for local release of drugs.

Conflict of interest statement

Apart from Mike de Leeuw and Esther de Boef of Ingell Labs BV, none of the authors of this paper have 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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