Influence of an intra-articular lipopolysaccharide challenge on markers of inflammation and cartilage metabolism in young horses

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ABSTRACT: Nineteen weanling Quarter Horses (225 to 380 kg) were used in a randomized complete block design to investigate the effects of intra-articular lipopolysaccharide (LPS) to induce acute joint inflammation in young horses. Horses were blocked by age, BW, and sex and were randomly assigned to 1 of 3 treatments for a 35-d experiment. Treatments included intra-articular injection of 0.25 ng (n = 7) or 0.50 ng (n = 6) of LPS obtained from Escherichia coli O55:B5 or sterile lactated Ringer’s solution (n = 6; control) into the radial carpal joint. Synovial fluid was obtained at preinjection h 0 and 2, 6, 12, 24, 168, and 336 h postinjection and was analyzed for PGE2, carboxypeptide of type II collagen (CPII), and collagenase cleavage neopeptide (C2C) biomarkers via commercial ELISA kits. Rectal temperature (RT), heart rate (HR), respiratory rate (RR), and carpal circumference were recorded before each sample. Lameness scores on a 0 to 5 scale were conducted after arthrocentesis. Data were analyzed using PROC MIXED procedure of SAS. Linear and cubic effects were tested in the form of contrasts. Clinical assessment of HR, RR, and RT were not influenced by treatment (P ≤ 0.16). All horses exhibited increased lameness scores over time (P ≤ 0.01), and horses receiving LPS, regardless of dose, had greater recorded lameness scores at 12 and 24 h postinjection (P ≤ 0.05). Joint circumference increased (P ≤ 0.01) across treatments in response to repeated arthrocentesis. Mean synovial fluid PGE2 concentrations increased linearly with increasing levels of LPS administration (P ≤ 0.01). Additionally, regardless of treatment, PGE2 increased over time and peaked at 12 h postinjection (P ≤ 0.01) and remained elevated above baseline at 336 h postinduction. Synovial concentrations of anabolic CPII increased linearly (P ≤ 0.01) with increasing dosage of LPS and increased (P ≤ 0.01) over 24 h in all horses, beginning at 6 h and peaking at 24 h postinjection. Concentrations of C2C in synovial fluid were not influenced by treatment and decreased from 0 to 6 h and steadily increased to 24 h in all horses (P ≤ 0.01). These results indicate that intra-articular LPS induced intra-articular inflammation and collagen synthesis in young horses and that the response is dose dependent. The use of this model to induce predictable joint inflammation may provide insight to the efficacy of preventative strategies relating to joint disease in the young horse.

Key words: cartilage, horse, inflammation, lipopolysaccharide

INTRODUCTION

Osteoarthritis (OA) is a significant cause of lameness in horses, which leads to the retirement of equine athletes (Todhunter and Lust, 1990). Adaptation of bone and soft tissue occurs during early exercise, when young horses undergo repeated trauma and stress, resulting in the overproduction of inflammatory mediators. Intra-articular lipopolysaccharide (LPS) injection is an established model for induction of localized inflammation in the mature horse (Ishihara et al., 2005; Lindegaard et al., 2010). In a recent study, synovial PGE2 was determined to be an important mediator of local inflammation and increased in response to intra-articular injection of 0.5 ng/mL solution (de Grauw et al., 2009). Synovial PGE2 has also been used as an indicator of OA in naturally occurring arthritis (Bertone et al., 2001).

When compared with dogs and other mammals, horses are considered to be 10 times more susceptible to LPS,
and it has been suggested that equine chondrocytes may be more responsive to LPS when compared with other species (MacDonald et al., 1994). This concentration of sensitivity is an important consideration for using this model to produce severe, but transient, joint inflammation in the horse. Intra-articular injection of LPS also influences the metabolism of articular cartilage as determined by biomarkers. Parallel profiles of catabolic collagenase cleavage neopeptide (C2C) and anabolic carboxypeptide (CPII) increased in response to LPS injection in mature horses (de Grauw et al., 2009). Although previous work has demonstrated the effectiveness of intra-articular LPS to induce inflammation and cartilage turnover in mature horses, there are limited data using a young-horse model. The objective of this study was to determine an appropriate intra-articular LPS dosage that could be used in the growing horse, as well as to investigate the effect of localized inflammation on cartilage metabolism in response to varying doses of LPS.

**MATERIALS AND METHODS**

All care, handling, and sampling of horses were approved by the Texas A&M University Institutional Animal Care and Use Committee.

**Horses and Treatments**

Nineteen Quarter Horse weanlings of similar breeding (initial BW of 225 to 380 kg; 184 to 327 d of age; 12 colts and 7 fillies) from the Texas A&M University herd were used in a randomized complete block design for a 35-d experiment. Horses were blocked on the basis of age, BW, and sex and were randomly assigned within block to 1 of 3 treatments. Treatments, based on previously reported data (Palmer and Bertone, 1994; de Grauw et al., 2009) in mature horses, included intra-articular injection of 0.25 ng (n = 7) or 0.50 ng (n = 6) of LPS solution obtained from *Escherichia coli* O55:B5 (Sigma-Aldrich, St. Louis, MO) or sterile lactated Ringer’s solution (LRS; n = 6; control). Purified LPS was reconstituted in LRS to achieve a stock solution of 1 mg/mL. Serial dilutions from the stock concentration were performed to yield individual doses of 0.8 mL as described previously by de Grauw et al. (2009).

For 21 d before induction of inflammation, horses were housed according to BW in 25 × 25 m dry lots (4 to 5 horses per pen) to centralize housing and standardize diet. Horses were group fed a commercially available 16% CP pelleted concentrate (Producer’s Cooperative Association, Bryan, TX) at 1% BW (as-fed basis) and 0.75% BW (as-fed basis) per day of coastal Bermuda grass hay (*Cynodon dactylon*). During adaptation, BW was recorded weekly, and group intake was adjusted accordingly.

**Sample Collection**

After adaptation (d 21), localized inflammation was induced to the left radial carpal joint. This joint was chosen for ease of use and sampling consistency. At preinjection h 0 (PIH 0), the carpus was clipped and aseptically prepared for arthrocentesis. Horses were restrained but not sedated for arthrocentesis. Carpal arthrocentesis was performed by veterinarians from the Texas A&M University Large Animal Clinic (College Station, TX). All treatments (0.8 mL solution) were administered aseptically at a location medial to the extensor carpi radialis tendon in the palpable depression between the radial carpal bone and the third carpal bone to a depth of approximately 12.7 mm to avoid unnecessary contact with articular cartilage (McIlwraith and Trotter, 1996).

Synovial fluid samples (1 to 4 mL) were obtained via arthrocentesis at PIH 0 and 2, 6, 12, 24, 168, and 336 h postinjection and were transferred into sterile non-additive tubes. Samples were immediately placed on ice, then divided into small aliquots and stored at -20°C for later analysis of PGE$_2$, CPII, and C2C. Before each synovial fluid sample, rectal temperature (RT), heart rate (HR), and respiratory rate (RR) were recorded. Joint circumference (cm) was determined at the level of the accessory carpal bone with a soft tape measure. American Association of Equine Practitioners (AAEP) lameness scores were assigned on a 0 to 5 scale and were conducted immediately after arthrocentesis (Ross, 2003). Horses were consistently evaluated within 2 min after arthrocentesis. The grade of lameness was determined at a trot, with grade 0 as sound and grade 5 as non–weight bearing, with all scores assigned by the same observer. All physical variables and lameness scores were determined throughout the first 24 h when all horses were observed continuously in individual 3.0 × 3.0 m stalls.

**Synovial Fluid Analysis**

Synovial fluid samples were analyzed for concentrations of PGE$_2$ using an ELISA (R&D Systems, Minneapolis, MN). This ELISA is designed to measure PGE$_2$ in human cell culture supernates, serum, plasma, and urine. However, there are no species differences in arachidonic acid derivative structure; therefore, the assay can be equally applied to equine samples (Bertone et al., 2001; de Grauw et al., 2006). Synovial fluid samples required a 1:4, 1:8, and 1:10 dilution depending on time postinjection. Dilutions were made with the calibrator diluents provided by the kit before beginning the assay. The mean minimum detectable dose was 30.9 pg/mL. Interassay precision ranged from 8.5% to 9.8%.

A commercially available ELISA kit (IBEX Pharmaceuticals Inc., Quebec, Montreal, Canada) was used to evaluate CPII concentrations in synovial fluid.
Standards were diluted (1:2) following kit recommendations, and samples were diluted (1:4) with an assay buffer solution provided by the manufacturer. Synovial fluid concentrations of C2C were also determined using a commercially available ELISA kit (IBEX Pharmaceuticals Inc.). Interassay precision ranged from 8.1% to 9.7% for C2C and 8.6% to 9.8% for CPII. Minimum detection limits for CPII and C2C were 50 and 10 ng/mL, respectively. Both of these assays have been previously validated for use in equine synovial fluid (Billinghurst et al., 2001; Frisbie et al., 2008). All plates were read (BioRad 680 Microplate Reader, BioRad Laboratories, Hercules, CA) at an optical density of 450 nm.

Data were analyzed using PROC MIXED procedure (SAS Inst. Inc., Cary, NC). The model contained effects for treatment, time, and treatment by time interaction. Linear and cubic effects were tested in the form of orthogonal contrasts. Block was included as part of the initial model; however, it was not significant for variables tested and was therefore removed. Significance differences were declared at \( P \leq 0.05 \), and \( P \leq 0.10 \) was considered a trend toward significance.

RESULTS

Physical Variables

Lameness scores were linearly influenced by treatment \( (P = 0.05) \) with horses receiving LPS injection (Fig. 1). Lameness scores also increased \( (P \leq 0.01) \) over time in response to repeated arthrocentesis, regardless of treatment. Similar to lameness scores, carpal circumference increased \( (P \leq 0.01; \text{Fig. 2}) \) across all treatments over 24 h; however, there was no influence \( (P = 0.96) \) of treatment on carpal circumference. Intra-articular LPS injection did not influence clinical outcomes, including HR (Fig. 3, \( P = 0.78 \)), RR (Fig. 4, \( P = 0.16 \)), and RT (Fig. 5, \( P = 0.60 \)), and all values remained within normal physiological limits. However, HR and RR decreased over time across treatments \( (P \leq 0.01) \), as horses became adapted to handling procedures. Rectal temperatures were influenced by time \( (P \leq 0.01) \) and peaked at 12 h postinjection in all horses, corresponding to peak levels of synovial inflammation, and decreased by 24 h.

Synovial Inflammation

Synovial PGE\(_2\) concentrations were linearly influenced \( (P \leq 0.01) \) by treatment, with PGE\(_2\) concentrations increasing in response to increasing intra-articular LPS administration (Fig. 6). There was also a treatment by time interaction \( (P \leq 0.01) \) at 2 and 12 h, with horses receiving 0.5 g of LPS solution having greater concentrations of PGE\(_2\), which peaked at 12 h postinjection, when compared with both the intermediate dose and control, respectively. Furthermore, there was an influence of time as all horses, regardless of treatment, had increased \( (P \leq 0.01) \) PGE\(_2\) concentrations, which peaked at 12 h postinduction and remained increased above baseline at 336 h in response to repeated arthrocentesis.

Cartilage Biomarkers

Catabolic C2C concentrations were not influenced \( (P = 0.24) \) by intra-articular treatment (Fig. 7). However, C2C concentrations were influenced \( (P \leq 0.01) \) over time as synovial C2C decreased from 0 to 6 h \( (269.99 \pm 22.64 \text{ ng/mL}) \) and peaked at 24 h \( (344.97 \pm 23.34 \text{ ng/mL}) \) postinjection. In contrast, anabolic CPII was linearly influenced by treatment \( (P \leq 0.01) \), as CPII concentrations increased with increasing levels of LPS injection, with the exception of 12 and 168 h postinjection, when the 0.25-ng

**Figure 1.** American Association of Equine Practitioners (AAEP) lameness scores (0 to 5; least squares means ± SEM) after intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 24 h postinjection. Treatments were control (lactated Ringer’s solution only; \( n = 6 \)), 0.25 ng LPS solution \( (n = 7) \), and 0.50 ng LPS solution \( (n = 6) \). Main effect is time \( (P \leq 0.01) \). *a,b* Different superscripts denote a difference \( (P \leq 0.05) \) among treatments.

**Figure 2.** Carpal circumference (cm; least squares means ± SEM) after intra-articular lipopolysaccharide (LPS; derived from *Escherichia coli* O55:B5) injection at 0 to 24 h postinjection. Treatments were control (lactated Ringer’s solution only; \( n = 6 \)), 0.25 ng LPS solution \( (n = 7) \), and 0.50 ng LPS solution \( (n = 6) \). *a–d* Different superscripts denote the main effect of time \( (P \leq 0.01) \).
dosage resulted in greater concentrations (Fig. 8). A treatment by time interaction ($P = 0.01$) was also observed at 6 and 24 h, with horses receiving the greatest LPS dosage having greater CPII concentrations when compared with both the intermediate dose and controls, which peaked at 24 h postinjection. In a more delayed response, the intermediate dose of 0.25 ng LPS resulted in greater CPII at 12 and 168 h postinjection when compared with the 0.5 ng LPS dosage and controls. However, it is important to note that CPII and C2C concentrations did not differ from baseline values ($P = 0.18$ and $P = 0.71$, respectively) at 336 h postexposure.

**DISCUSSION**

Clinical responses of HR, RR, and RT in the current study are similar to previously reported data in mature horses (Hawkins et al., 1993; de Grauw et al., 2009). Meulyzer et al. (2009) determined intra-articular injection of 0.25 and 0.50 ng LPS resulted in no signs of systemic illness, and any pain and lameness was resolved after 48 h in 8 mature horses ($14.4 \pm 2.1$ yr). Palmer and Bertone (1994) concluded that intra-articular LPS injection of 0.50 ng resulted in mild to moderate joint effusion and increased joint circumference of 1 to 1.5 cm above baseline, warmth, mild resistance to palpation, and a grade of lameness ranging from 1 to 3 at 12, 24, and 36 h postinjection. Also, horses (2 to 20 yr) did not exhibit systemic signs of illness, including increased peripheral temperature, HR, and RR (Palmer and Bertone, 1994).

In the current study, carpal circumference increased 1.21 cm above baseline ($28.39 \pm 2.09$ cm) over 24 h, which is indicative of joint effusion in response to repeated arthrocentesis. Similarly, lameness scores increased (0 to 1.5) over 24 h across treatments. Scores were greater in LPS-treated horses at 12 and 24 h postinjection, which corresponded to peak synovial PGE$_2$ concentrations. Findings by May et al. (1994) and Bertone et al. (2001) indicated that synovial fluid concentrations of PGE$_2$ are linked to grade of lameness and degree of joint pain in naturally occurring OA. Therefore, the current study using young horses agrees with previous experiments using mature horses and indicates that intra-articular LPS can be used as an experimental model to induce synovitis with minimal physiological changes (HR, RR, RT) and minor increase in lameness scores.

Prostaglandins, most notably PGE$_2$, assume an active role in articular inflammatory and nociceptive pathways (Kirker-Head et al., 2000). The metabolite PGE$_2$ is synthesized from arachidonic acid, with greater concentrations predominately associated with cells of the synovial membrane in contrast to articular cartilage, and is considered an indicator of active joint inflammation (McIlwraith and Trotter, 1996). Concentrations of synovial PGE$_2$ have been reported in nonarthritic joints at $36.5 \pm 12.0$ pg/mL, with increased concentrations of $573.50 \pm 314.00$ pg/mL in arthritic horses (Gibson et al., 1996; van den Boom et al., 2005). When inflamma-
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...tion is experimentally induced with LPS, concentrations of eicosanoids vary with time postinjection, with PGE$_2$, leukotriene B$_4$, and thromboxane A$_2$ increasing in the first 12 h and only PGE$_2$ remaining increased after 24 h (Gibson et al., 1996). de Grauw et al. (2009) reported a sharp and short-lived increase in PGE$_2$ at 8 h after intra-articular injection of 0.5 ng LPS solution in mature horses. In the current study, synovial PGE$_2$ peaked later at 12 h (2,541.72 ± 127.53 pg/mL) across treatments. This delayed increase could be a reflection of the more frequent number of samples obtained via arthrocentesis or a variation due to the younger model used in the present study.

The use of intra-articular LPS in nanogram quantities can mimic acute synovitis in mature or arthritic horses; however, this has yet to be fully elucidated in a young, growing model. Although not measured in the current study, Palmer and Bertone (1994) demonstrated synovial fluid total nucleated cell count and total protein were linearly responsive to increases in intra-articular LPS dosages of 0.125 up to 0.5 ng per joint in mature horses. Dosages greater than 0.5 ng per joint resulted in clinical signs of endotoxemia, including fever, depression, and non-weight-bearing lameness. At the 0.125-ng dose, horses exhibited mild to moderate effusion and warmth of the injected joint, mild resistance to palpation, and grade 2 lameness. However, injections were repeated at 48, 96, and 144 h to maintain the synovitis. Single dosages of 0.25 and 0.5 ng of LPS were chosen to prevent the need for multiple LPS injections and to determine the influence of LPS dosages commonly used in a mature model on young horses. Sham-injected control joints were not used in a previous study, and therefore, direct comparisons could not be established between effects of arthrocentesis alone on synovial fluid markers vs. effects of intra-articular LPS (de Grauw et al., 2009). The current study clearly indicates an influence of repeated arthrocentesis alone and demonstrates the importance of sham-injected controls.

Inflammation and articular cartilage degradation are considered important features in development of joint disease, and concentrations of cartilage biomarkers measured within synovial fluid can be influenced by local inflammatory status. Type II collagen is an important component to articular cartilage, and breakdown is key to the development of joint disease. Cartilage destruction leads to an accumulation of breakdown products in synovial fluid. Analysis of these fragments can provide valuable information regarding turnover of cartilage before gross pathology or can highlight any metabolic changes (Garvican et al., 2010).

The C2C antibody recognizes collagenase-cleaved type II collagen fragment and has been measured in mechanically induced OA in dogs, with concentrations increasing at 3 and 12 wk of age when compared with normal joints (Chu et al., 2002). The concentration of C2C increases with joint inflammation in rabbits and anterior cruciate transection in dogs, indicating it may serve as...
a useful biomarker relating to joint health (Fraser et al., 2003; Matyas et al., 2004). In the current study, concentrations of synovial C2C were not influenced by LPS injection; however, concentrations steadily increased from 6 to 24 h across treatments. de Grauw et al. (2009), using a 0.5-ng dosage and a similar arthrocentesis protocol, reported a similar rise in C2C in mature horses; however, peak values were 2 times greater than those in the current study, indicating concentrations of C2C may be influenced by age.

The CPII molecule is proteolytically cleaved from the procollagen strand during fibril formation, and concentration of this peptide has been directly related to the rate of collagen synthesis, which increases in arthritic joints (de Grauw et al., 2006). In the current study, concentrations of CPII increased with increasing LPS dosage, demonstrating a metabolic shift to cartilage synthesis to mend damage to the collagen framework in this young equine model. Because the half-life of the cleaved propeptide is relatively short (16 h) in synovial fluid, it is a good indicator of recent synthesis (Garvican et al., 2010).

In human clinical trials, CPII concentrations were greater in articular cartilage, serum, and synovial fluid from patients diagnosed with joint disease (Rizkalla et al., 1992; Nelson et al., 1994). Frisbie et al. (1999) reported CPII concentrations increased in response to osteochondral fragmentation in young exercising horses. de Grauw et al. (2009) induced synovitis using LPS in a mature model and observed CPII concentrations increased in a delayed manner at 24 h and remained elevated at 168 h after intra-articular injection. However, in the current study peak values at 24 h in the 0.5-ng dose horses were 2 times lower and corresponded more closely to previously reported data in exercising 2-yr-old horses induced with OA (Frisbie et al., 2008). This result suggests that variations in C2C and CPII between studies may be due to age and growth. Comparing mature-horse LPS data to the current growing model may not be accurate. Young horses have the greatest rate of cartilage formation and can easily replace damaged joint tissue (Brama et al., 2010). This corresponds to the current study, with the young model increasing the rate of synthesis rather than degradation.

In summary, carpal circumference and lameness scores increased over time across treatments and were attributed to repeated arthrocentesis. Horses receiving LPS had greater recorded lameness scores beginning at 12 h when compared with their control counterparts. Anabolic CPII was linearly influenced by treatment, and catabolic C2C increased over time across treatments. When measuring intra-articular inflammation, PGE$_2$ concentrations were linearly influenced by treatment and peaked at 12 h after injection. Therefore, the intra-articular LPS challenge was sufficient in inducing inflammation and cartilage turnover in a young-horse model. An increased dose of LPS resulted in increased concentrations of PGE$_2$ and CPII in synovial fluid, with no outward changes in physical status. Therefore, this study provides valuable information regarding the influence of varying dosages of LPS in the young horse, and this model could be effectively used in further studies to induce predictable levels of inflammation and alter cartilage metabolism.

**LITERATURE CITED**


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