Effects of sodium hyaluronate and methylprednisolone acetate on proteoglycan metabolism in equine articular chondrocytes treated with interleukin-1

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Objective—To determine the effects of sodium hyaluronate (HA) in combination with methylprednisolone acetate (MPA) on interleukin-1 (IL-1)–induced inflammation in equine articular cartilage pellets.

Sample Population—Chondrocytes collected from 7 horses euthanatized for problems unrelated to the musculoskeletal system.

Procedures—Chondrocyte pellets were treated with medium (negative control); medium containing IL-1 (positive control); or medium containing IL-1 with MPA (0.05 or 0.5 mg/mL), HA (0.05 or 0.5 mg/mL) or MPA (0.05 or 0.5 mg/mL) and HA (0.2 or 2 mg/mL) in combination. Proteoglycan (PG) synthesis was determined by incorporation of sulfur 35–labeled sodium sulfate into PGs. Glycosaminoglycan (GAG) content of the media and the pellets and total pellet DNA content were determined.

Results—Methylprednisolone acetate at 0.5 mg/mL caused an increase in PG synthesis, whereas HA had no effect alone. The combination of MPA, both 0.05 mg/mL and 0.5 mg/mL, with HA at 2 mg/mL increased PG synthesis, compared with IL-1–treated control. All treatment groups containing the high concentration of MPA (0.5 mg/mL) and the high concentration of HA (2.0 mg/mL) had pellets with increased GAG content. The addition of HA caused an increase in total GAG content in the media, regardless of MPA treatment. Cyclooxygenase-2 mRNA and aggrecan mRNA expression was significantly reduced with MPA treatment. Total pellet DNA content was unchanged by any treatment.


There are 9.2 million horses in the United States, over 70% of which are involved in recreation or showing.1 In performance horses, OA is a major source of debilitating pain, economic loss, and loss of athleticism.2–4 Degenerative joint disease is much more common and has a greater economic importance than acute traumatic injuries or respiratory diseases in performance horses.1,4 Several studies5–7 estimate that problems involving the fetlock and carpal joints account for 25% to 28% of the horses lost from training. In dressage horses, degenerative joint disease of the distal hock joints, forelimb pastern joints, and fetlock joints is a common cause of reduced performance.1 This project is designed to evaluate the benefits and potential adverse effects of commonly used intra-articular medications given to performance and show horses.

Nutraceuticals, such as glucosamine and chondroitin sulfate, have been popularized for the treatment of OA. However, oral supplementation alone is generally ineffective in treating clinical lameness in athletic horses where the goal of treatment is to resolve the lameness while keeping the horse in work. Intra-articular injections of corticosteroids and systemically administered nonsteroidal anti-inflammatory agents remain the mainstays of treatment for relief of OA pain in equine athletes with OA.6–8 Intra-articular administration of corticosteroids rapidly resolves joint effusion, synovial inflammation, and articular pain. However, it remains controversial whether repeated use of corticosteroids, specifically MPA, can actually exacerbate the progression of OA while masking the clinical signs of disease.

In 1 study9 of abnormal joints, although corticosteroid treatment improved outward signs of joint inflammation, more articular cartilage degeneration and local bone necrosis were associated with corticosteroid–injected joints. Similar cartilage degeneration is seen with MPA in rabbits with experimentally induced osteochondral defects of the stifle joints.10 In exercising horses with experimentally created osteochondral fragments, treatment with MPA has a detrimental effect on articular cartilage.11 However, more recently, detrimental effects on articular cartilage were not consistently observed in horses with similarly created osteochondral fragments when betamethasone or triamcinolone was administered in separate studies, suggesting that MPA treatment may be more detrimental to articular cartilage.12 After weekly injections of MPA (120 mg) into

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normal equine carpal joints, chondrocyte necrosis and hypocellularity are found histologically, and reduced rates of PG and collagen synthesis are observed.13 The in vitro effect of MPA on osteoarthritic cartilage can be markedly different from normal cartilage.14 In low doses, MPA can have a protective effect on PG synthesis of osteoarthritic cartilage by reducing the inflammatory cascade.15 Conversely, higher doses of MPA have a profound detrimental effect on normal and osteoarthritic cartilage PG metabolism.13,15 To our knowledge, no studies have documented the effect of HA and MPA on PG synthesis of osteoarthritic or IL-1-treated cartilage.16–18 These findings suggest that exogenously administered HA compensates for the catabolic effects in joints treated with corticosteroids.19,20 Despite the frequent concurrent use of intra-articular corticosteroids and HA, to our knowledge, no in vitro studies of the effects of the combination on PG metabolism in experimentally inflamed or osteoarthritic joints have been published.

We recently evaluated the effect of HA and corticosteroids on normal equine articular cartilage explants.13 In that study, MPA caused a decrease in PG synthesis, whereas HA alone had no effect. Only the combination of MPA (0.05 mg/mL) and HA at a concentration of 1.0 mg/mL increased PG synthesis, compared with control explants. The purpose of the study reported here was to evaluate the effect of MPA and HA, alone or in combination, on IL-1-induced inflammation in equine articular cartilage pellets. Interleukin-1 has been used in articular cartilage to simulate OA.20,21 We tested the hypothesis that HA alone or in combination with corticosteroid administration can mitigate cartilage PG catabolism caused by IL-1 administration in equine chondrocytes pellets.

Materials and Methods
Pellet culture—Horses used for this study were euthanatized by use of a lethal dose of sodium pentobarbital administered IV for reasons unrelated to musculoskeletal disease. The Institutional Animal Care and Use Committee at the University of Illinois approved this study. Articular cartilage was aseptically collected from the metacarpal- or metatarsophalangeal ( fetlock) joints of 7 horses, ages ranging from 1 to 4 years. Cartilage was collected in PBS solution with 1% penicillin-streptomycin and ascorbic acid (50 μg/mL).2 Media were divided into 0.5-mL aliquots and placed in 1.5-mL Eppendorf tubes, then centrifuged to form chondrocyte pellets containing 250,000 cells/pellet. Pellets were incubated at 37°C in 5% CO2 for 7 days to allow the formation of an extracellular matrix. Media were changed every other day until treatments were performed.

On day 7, 10 pellets for each treatment group were supplemented with fresh media only (negative control; 1 treatment group) or media containing IL-1 only (10 ng/mL; positive control; 1 treatment group); IL-1 (10 ng/mL) and MPA (0.05 mg/mL or 0.5 mg/mL; 2 treatment groups); IL-1 (10 ng/mL) and HA (0.2 mg/mL or 2 mg/mL; 2 treatment groups); or IL-1 (10 ng/mL) with HA (0.2 mg/mL or 2 mg/mL) and MPA (0.05 mg/mL or 0.5 mg/mL; 4 treatment groups). This provided a total of 10 treatment groups including the controls. The concentrations of MPA and HA were determined from a range of concentrations of drug likely present in the fetlock joint within 24 hours after in vivo intra-articular injection.22–24 At the time of media change, 5 pellets from each treatment group were radiolabeled with media containing sulfur 35 (35S)–labeled sodium sulfate (10 μCi/mL). After 24 hours in treatment media, pellets were removed from culture conditions, washed in PBS solution, and stored at –80°C for later analysis of newly synthesized PG, total GAG content, and total DNA and quantitative PCR analysis of pellet mRNA. At least 1 pellet from each treatment group was frozen in optimal cutting temperature compound for cryosection and histologic examination. The exhausted media were also collected and stored at –80°C to determine GAG released into the media.

PG synthesis—New PG synthesis was determined by 35SO4 incorporation into the pellet PG content over a 24-hour period. Radiolabeled pellets were digested in 100 μL of papain (150 μg/mL) at 65°C for 24 hours. Aliquots of 25 μL of 35S-labeled papain-digested pellets were placed in multi-well punch plates, precipitated with Aicin blue dye, and counted by scintillation.25 All values were adjusted for radioisotope decay, and pellets were adjusted for the total volume of papain digestion added to the pellet.

Total GAG content of pellets and media—Exhausted media were placed into 100-μL aliquots and digested in papain (7.5 mg/mL) for 4 hours at 65°C. A dimethylmethylene blue assay was performed on all papain-digested pellets and media as previously described.26 All samples were compared against a standard curve of chondroitin sulfate to estimate the total GAG content of digested pellets and media. All samples were run in duplicate. Pellet data were adjusted for total volume of digestion. Media were also adjusted for total volume supplementing each pellet.

Total pellet DNA content—Total DNA content of the pellets was determined by use of a fluorometric assay and dye with 10-μL aliquots and a microplate reader.27 Samples were run in duplicate, compared with a standard curve of calf thymus DNA,28 and corrected for total volume of pellet digested.

Real-time RT-PCR analysis—Real-time RT-PCR data were obtained on 3 of the 7 horses. The RNA was extracted from 4 pellets from each treatment group (for a total of 1 million cells) by use of Trizol reagent with the manufacturer’s suggested protocol. Complementary DNA was generated by reverse transcriptase, first priming with oligo d(T). Real-time quantitative PCR analysis was performed in duplicate for COX-2 and aggrecan, normalized to elongation factor-1α mRNA expression.29 A PCR detection system was used to perform the assay.

Statistical analysis—Data are presented as mean ± SD values. Significance was determined for IL-1, MPA, and HA by use of repeated-measures ANOVA with a software program.4 Post hoc tests were conducted when indicated by use of Bonferroni adjusted P values. Values of P ≤ 0.05 were con-
sidered significant. Non-normally distributed data (DNA content) were ranked to determine significance. If treatment with MPA, HA, or the combination of MPA and HA was significant, a 3-way multiple comparison was also made for individual treatments to the positive control (IL-1 treatment only), the MPA negative control (same concentration of HA with no MPA), and HA negative control (same concentration of MPA with no HA). For these comparisons, a value of \( P < 0.001 \) was considered significant to account for experiment-wise error rates that occur with multiple comparisons.

**Results**

**Pellet PG synthesis**—Treatment with IL-1 significantly \( (P = 0.01) \) decreased PG synthesis, causing a 2.3-fold decrease when the negative control (no IL-1) was compared with the positive control (IL-1 treatment only; Figure 1). Synthesis of PG was significantly \( (P = 0.045) \) affected by HA treatment. In addition, combined treatment with MPA and HA had a significant \( (P < 0.001) \) interaction on PG synthesis, whereas MPA treatment alone had no significant \( (P = 0.082) \) effect on PG synthesis. The high concentration of HA (2 mg/mL) combined with MPA (0.05 and 0.5 mg/mL) significantly increased PG synthesis, compared with IL-1–treated controls, by 2.4-fold and 2.7-fold, respectively. The addition of MPA at 0.5 mg/mL caused a 2.3-fold increase in PG synthesis, compared with the IL-1 treated control.

**Total pellet GAG content**—Treatment with IL-1 had no significant \( (P = 0.709) \) effect on total pellet GAG content when the negative control (no IL-1) was compared with the positive control (IL-1 treatment only; Figure 2). Separately, HA \( (P = 0.034) \) and MPA \( (P = 0.001) \) treatment had a significant effect on total pellet GAG content. Combined treatment with MPA and HA revealed no significant \( (P = 0.165) \) interaction on total pellet GAG content. The addition of HA at 2.0 mg/mL alone or in combination with MPA resulted in a significantly higher GAG content of the pellet, ranging from a 1.6- to 2.0-fold increase, compared with IL-1–treated controls. The addition of MPA at 0.5 mg/mL alone or in combination with HA produced a significant change in GAG content in the pellet, ranging from a 1.8- to 2.0-fold increase, compared with IL-1 treated controls.

**PG synthesized and released into the media**—Treatment with IL-1 had no significant \( (P = 0.539) \) effect on the PG synthesized and released into the media when the negative control (no IL-1) was compared with the positive control (IL-1 treatment only; Figure 3). Supplementation of the pellets with HA alone \( (P = 0.245) \), MPA alone \( (P = 0.237) \), or in combination \( (P = 0.686) \) had no significant effect on total PG synthesized and released into the media, compared with positive controls (IL-1 treatment only).

**Total GAG concentration in media**—Treatment with IL-1 had no significant \( (P = 0.669) \) effect on total GAG concentration in the media when the negative control (no IL-1) was compared with the positive control (IL-1 treatment only; Figure 4). The addition of HA resulted in a significant \( (P < 0.001) \) increase in total GAG content in the media, compared with IL-1–treated controls. Content of GAG in the media was unaffected \( (P = 0.197) \) by MPA treatment, and no significant \( (P = 0.595) \) interaction was found with cotreatment of MPA and HA. Treatment with HA at 0.2 mg/mL and 2.0 mg/mL resulted in a 1.5- to 1.9-fold increase in GAG concentration in the media, compared with IL-1–treated controls.

![Figure 1](image1.png)  
**Figure 1**—Mean ± SD incorporation of sulfur 35–labeled sodium sulfate into GAG of equine articular chondrocyte pellets treated with various concentrations of MPA and HA. Scintillation counts (counts per minute [CPM]) were normalized for decay and pellet digestion volume. *Significant \( (P < 0.05) \) difference between negative control (no IL-1) and positive control (IL-1 treatment only). †Significant \( (P < 0.001) \) difference between combined treatment with HA and MPA and positive control (IL-1 treatment only). ‡Significant \( (P < 0.001) \) effect of MPA, compared with no MPA (0 mg/mL), at the same concentrations of HA. §Significant \( (P < 0.001) \) effect of HA, compared with no HA (0 mg/mL), at the same concentrations of MPA.

![Figure 2](image2.png)  
**Figure 2**—Mean ± SD total GAG content in equine articular chondrocyte pellets treated with various concentrations of MPA and HA. Content of GAG was normalized for pellet digestion volume. See Figure 1 for remainder of key.
increase in GAG content in the media, compared with IL-1-treated controls, irrespective of MPA treatment.

Total pellet DNA content—Treatment with IL-1 had no significant \((P = 0.984)\) effect on total pellet DNA content when the negative control (no IL-1) was compared with the positive control (IL-1 treatment only; Figure 5). Total pellet DNA content was not significantly affected by HA alone \((P = 0.637)\), MPA alone \((P = 0.420)\), or cotreatment with HA and MPA \((P = 0.445)\).

Pellet mRNA expression—Real-time RT-PCR analysis revealed a significant increase (34.3-fold) in the COX-2 mRNA expression in IL-1–treated pellets, compared with negative controls (no IL-1; Figure 6). Treatment with HA alone had no suppressive effect on IL-1–stimulated COX-2 mRNA expression. However, MPA treatment caused a marked suppression in COX-2 mRNA expression at both concentrations studied, returning them to negative control (no IL-1) values.

Aggrecan mRNA expression mirrored COX-2 mRNA expression (Figure 7). The IL-1–treated pellets had a 3.6-fold increase in aggrecan mRNA expression over negative control (no IL-1) values. Supplementation with HA alone provided no further significant increase in aggrecan mRNA expression. In all treatment groups containing MPA, aggrecan mRNA expression was returned to negative control (no IL-1) values.

Discussion

Our study elucidated beneficial effects of HA alone and in combination with MPA on IL-1–stimulated chondrocytes pellets. Hyaluronic acid increased total GAG content of the pellets and media. Treatment with MPA was beneficial in retaining pellet GAG and reducing COX-2 mRNA expression in IL-1–treated chondrocyte pellets. Combination treatment yielded an increase in total pellet GAG and new PG synthesis, compared with the IL-1–treated controls.

Although HA alone had no effect on new PG synthesis, the combination of high-concentration HA (2 mg/mL) with low-concentration MPA (0.05 mg/mL) mitigated the detrimental effects of IL-1. The magnitude of this effect was similar to that of high-concentration MPA (0.5 mg/mL) alone and in combination with high-concentration HA (2 mg/mL). This suggests that in an inflammatory environment, higher concentrations of HA may act in an additive or synergistic fashion.
with lower concentrations of MPA to protect new PG synthesis by chondrocytes. Further benefits may not be achieved when HA is combined with higher concentrations of MPA.

Unexpectedly, aggrecan mRNA expression results did not mirror new PG production data, but rather resembled the COX-2 mRNA expression results. This may be the result of increased metabolism (enhanced aggrecan mRNA expression) in concert with increased catabolism (enhanced COX-2 mRNA expression) in response to IL-1 stimulation. The disparity between aggrecan mRNA expression data and new PG production may be attributed to the fact that new PG production includes products other than aggrecan.

Treatment with IL-1 upregulated mRNA expression of the inflammatory enzyme COX-2, potentially contributing to matrix degradation. Like other corticosteroids, MPA works by inhibiting leukocyte movement and function. Corticosteroids also influence the humoral aspect of inflammation by binding to steroid-specific receptors that modulate gene transcription of proteins involved in inflammation. In addition to downregulation of other inflammatory mediators, corticosteroids inhibit phospholipase A2 production. Phospholipase A2 production stimulates the arachidonic acid inflammatory cascade, leading to increases in inducible COX-2 and constitutive COX-2. Through the downregulation of the inflammatory cascade, MPA treatment returned COX-2 mRNA expression back to negative control (no IL-1) values. As shown by the total pellet GAG content data, MPA treatment protected the PG matrix. This may be attributable to its potent anti-inflammatory effect. In contrast, HA had no effect on COX-2 mRNA expression, suggesting that the benefits of concurrent MPA-HA treatment are attributable to effects other than just abrogation of the inflammatory cascade.

The total GAG content of the media increased significantly with the addition of HA, regardless of MPA treatment. This may partially be attributed to assay interference from the HA added to the media that was not processed by the pellet. Following this finding, an HA dose-response curve for the dimethylmethylene blue assay was performed, revealing that the addition of HA increased the total GAG content reading of the media. New PG production, as assayed by radiolabeled 35SO4, is not prone to such interference. Therefore, we consider the new PG production data more accurate. No difference was found in the amount of newly synthesized PG released into the media with HA treatment.

The total GAG content in the pellet was the most important datum. This represents the PG synthesized and the PG retained in the pellet despite IL-1 stimulation. In our study, a significant beneficial effect was found with HA and MPA treatment, alone and in combination, on total pellet PG content. The predominant benefit of MPA treatment was likely due to counteraction of the inflammatory effects of IL-1. The increase in retained pellet GAG seen with HA treatment in our study could not completely be explained by an increase in PG synthesis or a reduction in loss of newly synthesized PG. It is likely that exogenous supplementation with HA could have abrogated the loss of HA from the matrix that occurs with IL-1 treatment. Preventing HA loss from the matrix would minimize the loss of PGs attached to HA within the pellet matrix.

As with previous experiments in our laboratory, intermediate doses of MPA did not cause a significant reduction in DNA content. Although the DNA content of the pellets was not significantly changed by MPA treatment, we observed variability within individual responses at the MPA concentration of 0.5 mg/mL. Three of 7 horses had a marked decline in pellet DNA.

**Figure 6**—Mean ± SD COX-2 mRNA of equine articular chondrocyte pellets treated with various concentrations of MPA and HA. The mRNA values obtained by real-time RT-PCR analysis were normalized with elongation factor-1α. See Figure 1 for remainder of key.

**Figure 7**—Mean ± SD aggrecan mRNA of equine articular chondrocyte pellets treated with various concentrations of MPA and HA. The mRNA values obtained by real-time RT-PCR analysis were normalized with elongation factor-1α. See Figure 1 for remainder of key.
content with MPA at 0.5 mg/mL. Furthermore, in the first horse, a dose-response curve was performed with an MPA concentration of 5.0 mg/mL. At the MPA concentration of 5.0 mg/mL, no discernible DNA content was detected. This concentration of MPA was detrimental regardless of the addition of HA and was subsequently deleted from the experiment. Other histologic studies have shown that high doses of MPA (5 to 10 mg/mL of medium) are toxic to chondrocytes. An important variable to consider is the molecular weight of HA used. Our study evaluated a medium–molecular-weight HA (500,000 to 730,000) on the basis of a large analysis that suggested medium–molecular-weight HA might be the most therapeutic for intra-articular administration. It should be noted that a different molecular weight may yield different results than reported here. Results of some studies indicate that higher molecular weights of HA may be more beneficial.

In contrast to the current study, in a previous study from our laboratory, we did not document any beneficial effects of MPA. This can be attributed to the fact that the prior study was conducted in a noninflammatory model, and treatment with MPA is primarily beneficial in situations of inflammation. Also in contrast to the prior study, results of the present study indicate that cotreatment with MPA and HA increases retention of pellet GAG content and increases pellet PG synthesis, compared with IL-1-treated controls. This result is in contrast with the previous study, in which HA alone had no effect and HA in combination with MPA had minimal effect on PG metabolism. Some of the difference between studies can be attributed to response differences between conditions of inflammation and noninflammation. In addition, marked variation in GAG content of cartilage explants occurred between horses and within individual horses in the previous study that may have precluded detecting a small difference. The present study used a chondrocyte pellet model rather than explants in an effort to reduce the variability in total GAG content of the matrix.

In our study in which we evaluated the total pellet PG content, the high concentration of HA in combination with MPA seemed to be the most beneficial to PG matrix metabolism in the face of IL-1 treatment. Any additive or synergistic effects of concurrent treatment are the result of benefits to PG metabolism and not solely the result of inflammatory blockade. Findings in our study revealed the beneficial effects of MPA-HA cotreatment on chondrocyte pellets in an inflammatory environment. Future studies may be useful in evaluating the effects of other molecular weights of HA and types of corticosteroids.

References


