Effects of sodium hyaluronate and methylprednisolone acetate on proteoglycan synthesis in equine articular cartilage explants

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Objective—To determine effects of sodium hyaluronate (HA) on corticosteroid-induced cartilage matrix catabolism in equine articular cartilage explants.

Sample Population—30 articular cartilage explants from fetlock joints of 5 adult horses without joint disease.

Procedure—Articular cartilage explants were treated with control medium or medium containing methylprednisolone acetate (MPA; 0.05, 0.5, or 5.0 mg/mL), HA (0.1, 1.0, or 1.5 mg/mL), or both. Proteoglycan (PG) synthesis was measured by incorporation of sulfur 35-labeled sodium sulphate into PGS, and PG degradation was measured by release of radiolabeled PGs into the medium. Total glycosaminoglycan (GAG) content in media and explants and total explant DNA content were determined.

Results—Methylprednisolone acetate caused a decrease in PG synthesis, whereas HA had no effect. Only the combination of MPA at a concentration of 0.05 mg/mL and HA at a concentration of 1.0 mg/mL increased PG synthesis, compared with control explants. Methylprednisolone acetate increased degradation of newly synthesized PGs into the medium, compared with control explants, and HA alone had no effect. Hyaluronate had no effect on MPA-induced PG degradation and release into media. Neither MPA alone nor HA alone had an effect on total cartilage GAG content. Methylprednisolone acetate caused an increase in release of GAG into the medium at 48 and 72 hours after treatment. In combination, HA had no protective effect on MPA-induced GAG release into the medium. Total cartilage DNA content was not affected by treatments.

Conclusions and Clinical Relevance—Our results indicate that HA addition has little effect on corticosteroid-induced cartilage degradation and release into media.

Diseases of the musculoskeletal system are the leading cause of poor performance, economic loss, and lost training days in the equine athlete.1,2 Traumatic injury to articular cartilage, subchondral bone, and surrounding soft tissues leads to progressive changes of osteoarthritis (OA).3 Several medical treatments have been advocated to prevent OA or decrease clinical signs to allow continued competitive use of equine athletes. These treatments are directed toward decreasing inflammation in the diseased joint and thereby providing pain relief. Intra-articular administration of corticosteroids rapidly resolves the joint effusion, synovitis, and articular pain associated with OA and remains the mainstream of treatment in equine athletes.4-6 However, considerable controversy exists over the relative benefits of intra-articular corticosteroid administration. Results of several studies4,10-13 in horses indicate that the intra-articular administration of the common corticosteroid methylprednisolone acetate (MPA) is associated with deleterious effects on articular cartilage, while potentially masking clinical signs of disease. Corticosteroids impair chondrocyte metabolism, resulting in an altered biochemical composition, structural morphology, and mechanical integrity of articular cartilage.5,10-15

Intra-articular administration of sodium hyaluronate (HA) represents an alternative treatment for joint disease with an inflammatory component and is often used in conjunction with intra-articular administration of corticosteroids.16-18 Alone, HA has anti-inflammatory properties that have been demonstrated in vitro.19,20 Hyaluronate has dose- and molecular-weight-dependent inhibitory effects on prostaglandin E2 synthesis by synoviocytes and chondrocytes.21,22 Additionally, effects of interleukin-1-mediated proteoglycan (PG) degradation of articular cartilage have been mitigated by HA in vitro.23 Results of early studies16,17 in horses indicate that synovial cartilage degradation products are decreased when HA is combined with corticosteroid, compared with the use of corticosteroid alone. Furthermore, the loss of HA from cartilage through decreased synthesis or increased catabolism can result in a decrease in extracellular matrix PG retention.24,25 These findings suggest that exogenously administered HA may compensate for detrimental effects of corticosteroid administration in joints.

The purpose of the in vitro study reported here was to evaluate whether HA administration could mitigate the detrimental effects of a commonly used corticosteroid, MPA, on PG metabolism of equine articular chondrocytes. Our hypotheses were as follows: MPA treatment of equine articular cartilage explants would...
have negative effects on cartilage explant metabolism in a dose-dependent manner by decreasing matrix PG synthesis and increasing matrix PG degradation. HA treatment of equine articular cartilage explants would have positive effects on cartilage explant metabolism by increasing PG synthesis and decreasing PG degradation, and the addition of HA to MPA-treated cartilage would mitigate the negative effects of MPA on cartilage explant metabolism in a dose-dependent manner.

**Materials and Methods**

**Explant culture**—Articular cartilage was aseptically collected as full-thickness noncalcified explants from the fetlock joints of 5 adult horses between the ages of 2 and 11 years, within 1 hour of euthanasia for reasons unrelated to OA of the fetlock joint. A 6-mm-diameter punch biopsy instrument was used to maintain uniformity in size and approximate uniformity in weight of each explant. One explant from each fetlock joint was placed in 4% paraformaldehyde for 24 hours at 4°C prior to immersion in PBS solution. Histologic examination was performed to ensure the presence of baseline articular cartilage that was normal in histologic appearance. Explants were washed in PBS solution with 2% penicillin-streptomycin solution and placed in individual wells under sterile conditions within 2 hours of collection. One milliliter of Dulbecco modified Eagle medium containing 4.5 g of glucose, 10 mmol/L of sodium pyruvate, 1% penicillin-streptomycin, 1% L glutamine, 50 μg of ascorbic acid, and 10% fetal bovine serum were added to each well, and explants were incubated at 37°C in 5% CO₂ and 95% humidity for 24 hours to adapt to culture conditions.

Once adapted to culture conditions, explants were washed 3 times in PBS solution and placed in fresh medium (control) or medium containing MPA, HA, or MPA and HA in varying concentrations to create 16 treatment groups in replicates of 3 on day 0. The concentration of MPA (0.05, 0.5, or 5.0 mg/mL) and HA (0.1, 1.0, or 1.5 mg/mL) was established from concentrations of drug likely present in the fetlock joint within 24 hours after in vivo intra-articular injection. ²⁷ After 24 hours of treatment (day 1), the treatment medium was removed and explants were washed 3 times in PBS solution and placed into fresh medium containing no MPA or HA. At the end of the experiment (day 3), all media and explant samples were collected and stored at –80°C until total DNA and total glycosaminoglycan (GAG) isolation and PG analyses could be performed.

**PG synthesis and degradation**—At the time of treatment removal (day 1), all 3 replicates from each group were radiolabeled for 24 hours with fresh medium containing sulfur 35 (³⁵S)-labeled sodium sulphate (10 μCi/mL). The radiolabeled medium was collected at day 2 and stored at –80°C for baseline PG analysis. Explants were washed 3 times in PBS solution and returned to fresh media. All radiolabeled explants and media were collected at day 3 and stored at –80°C until PG analysis. At this time, radiolabeled explants were lyophilized, weighed, and digested in 0.5% papain at 65°C for 6 hours. ²⁷ Aliquots of 100 μL of ³⁵S-labeled papain-digested explants and radiolabeled media were placed in scintillation vials, and incorporation of ³⁵S into GAG in the cartilage and exhausted medium was determined by scintillation counting. All values were adjusted for radioisotope decay, and explants were adjusted for sample dry weight.

**Total GAG content of cartilage and media**—Exhausted media were placed into 100-μL aliquots and digested in papain (7.5 mg/mL) at 65°C for 4 hours. A dimethylmethylene blue dye-binding assay was performed on all papain-digested explants and media. All samples were compared against a standard curve on the basis of chondroitin sulfate to estimate the total GAG content of digest explants and media. ³⁷ All samples were run in duplicate, and explants were adjusted for sample dry weight.

**Total explant DNA content**—Total DNA content in explants was determined by use of a fluorometric assay and dye by reported methods. ³⁸ Samples were run in duplicate, compared with a standard curve, and normalized to tissue dry weight.

**Histologic evaluation**—Cartilage explants from each fetlock joint were placed in 4% paraformaldehyde for 24 hours before immersion in PBS solution at 4°C. Samples were dehydrated in alcohol, embedded in paraffin, and cut into 6-μm-thick sections. Routine H&E and safranin-O stains were used to ensure that no pathologic changes existed in the articular cartilage before beginning the study.

**Statistical analysis**—Data are presented as mean (± SD) values. Significance was determined by use of repeated-measures ANOVA with a software program. ³⁹ Non-normally distributed data were log transformed or ranked before an ANOVA was performed. Post hoc tests were conducted when indicated by use of Bonferroni adjusted P values. Values of P ≤ 0.05 were considered significant.

**Results**

**PG synthesis**—All concentrations of MPA resulted in a significant dose-dependent decrease in PG synthesis, compared with the control group (Figure 1). No significant difference was found in PG synthesis with the addition of HA at any concentration, compared with the control group. When MPA was added at a low concentration (0.05 mg/mL), concurrent administration of HA at 1.0 mg/mL caused a small but significant increase in new PG synthesis, compared with treatment with MPA at 0.05 mg/mL alone. No significant difference was found in PG synthesis between the low-dose MPA-treated groups and any other concentration of HA. No significant difference was found in PG synthesis between the medium-dose MPA-treated groups and the addition of HA at any concentration.

**PG degradation**—An MPA concentration of 5.0 mg/mL resulted in a significant decrease in the
release of newly synthesized PG, compared with the control group (Figure 2). No significant difference was found in the release of newly synthesized PG, compared with the control group, at any concentration of HA. The addition of HA at any concentration had no significant effect on the MPA-induced decrease in degradation of newly synthesized PG. Although treatment with MPA resulted in a significant decrease in the release of newly synthesized PG into the medium, the total amount of newly synthesized PG (retained in the cartilage and released into the medium) was decreased with MPA treatment. When the amount of newly synthesized PG released into the medium was expressed as a percentage of total new PG, MPA caused a dose-dependent increase in the percentage of newly synthesized PG released into the medium (Table 1).

Total cartilage GAG content—The total cartilage explant GAG content at any concentration of MPA did not significantly differ from the control group (Figure 3). The total cartilage explant GAG content at any concentration of HA did not significantly differ from the control group. However, when combined, the high-dose (5.0 mg/mL) MPA-treated group with the addition of HA at 1.0 mg/mL had a small but significant increase in the total GAG content of cartilage explants. No significant difference was found in any other MPA-treated groups with the addition of HA at any concentration.

Total GAG released into the medium—The GAG content in the medium was significantly increased by MPA. Specifically, MPA concentrations of 0.05 and 5.0 mg/mL resulted in a small but significant increase in the total release of GAG into the medium, compared with the control group, at day 2 (Figure 4). By day 3, a significant increase was found in the total release of GAG into the medium with MPA concentrations of 0.05, 0.5 and 5.0 mg/mL, compared with the control group (Figure 5). At days 2 and 3, no significant difference was found in the total GAG content of the medium, compared with the control group, at any HA concentration. No significant difference was found with the addition of HA at any concentration in the

Table 1—Percentage of newly synthesized proteoglycan (PG) released into the medium of equine articular cartilage explants treated with various concentrations of methylprednisolone acetate (MPA) and hyaluronic acid (HA).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PG synthesis</th>
<th>PG released</th>
<th>Total PG</th>
<th>PG released (%)</th>
</tr>
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<tr>
<td>No MPA</td>
<td>No HA</td>
<td>50,873</td>
<td>1,636</td>
<td>52,509</td>
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<tr>
<td></td>
<td>HA (0.1 mg/mL)</td>
<td>45,918</td>
<td>1,450</td>
<td>47,368</td>
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<td>HA (1 mg/mL)</td>
<td>49,019</td>
<td>1,531</td>
<td>50,550</td>
</tr>
<tr>
<td></td>
<td>HA (1.5 mg/mL)</td>
<td>49,627</td>
<td>1,738</td>
<td>51,365</td>
</tr>
<tr>
<td>MPA (0.05 mg/mL)</td>
<td>No HA</td>
<td>22,433</td>
<td>866</td>
<td>23,401</td>
</tr>
<tr>
<td></td>
<td>HA (0.1 mg/mL)</td>
<td>24,562</td>
<td>1,308</td>
<td>25,872</td>
</tr>
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<td></td>
<td>HA (1 mg/mL)</td>
<td>37,245</td>
<td>1,154</td>
<td>38,399</td>
</tr>
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<td></td>
<td>HA (1.5 mg/mL)</td>
<td>32,143</td>
<td>1,372</td>
<td>33,515</td>
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<tr>
<td>MPA (0.5 mg/mL)</td>
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<td>1,142</td>
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<td>892</td>
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<td>HA (0.1 mg/mL)</td>
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<tr>
<td></td>
<td>HA (1 mg/mL)</td>
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<td>1,345</td>
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<tr>
<td></td>
<td>HA (1.5 mg/mL)</td>
<td>331</td>
<td>1,175</td>
<td>1,506</td>
</tr>
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</table>

*Mean counts per minute of sulfur 35 (35S)-labeled papain-digested explants and media.
MPA-treated groups, compared with similar concentrations of MPA alone.

Total cartilage DNA content—Total cartilage DNA content was not significantly altered by MPA, compared with the control group, at any concentration (Figure 6). Total cartilage DNA content was not signifi-

![Figure 6](image_url)

*Figure 6—Mean (± SD) total DNA content in equine articular cartilage explants treated with various concentrations of MPA and HA. Content of DNA was normalized for dry tissue weight and log transformed for analysis. As with previous studies, results of our study provide evidence that treatment of articular cartilage with MPA is potentially detrimental to matrix metabolism. Specifically, MPA treatment decreased PG synthesis and increased cartilage explant GAG release into the medium. However, we did not see a corresponding decrease in GAG content in the explant itself. This may have been a result of the large variability found within individual explants.*

**Discussion**

Our hypothesis that the addition of HA to MPA-treated cartilage would mitigate the negative effects of MPA on cartilage explant metabolism was largely not supported by results of our study. Addition of HA at 1 mg/mL to MPA-treated explants did yield small positive effects on matrix metabolism in regard to PG synthesis and total explant GAG content. Because all other treatment groups had no change, compared with the control group, it is possible that these results are spurious. Further investigation into the effect of HA at 1 mg/mL on MPA-treated cartilage is necessary to support these positive findings.

The HA alone or in the face of MPA appeared to have a minimal effect on cartilage PG matrix metabolism because minimal or no change was observed, compared with the control group, with the addition of HA in regard to PG synthesis or degradation, total GAG content of the cartilage, total release of GAG into the medium, or DNA content of explants. Results of a previous study on cartilage affected by OA indicate that HA enhances PG synthesis and decreases the production and activity of proinflammatory mediators and matrix metalloproteinases, thereby decreasing matrix degradation. It may be necessary to use cartilage from a joint affected with OA to demonstrate the beneficial effects of HA on cartilage matrix metabolism through an anti-inflammatory mechanism. The anti-inflammatory properties of HA are well documented. As a result of the anti-inflammatory effects of HA, it may be necessary to have the presence of an inflammatory mediator, such as interleukin-1, to reveal a positive effect on cartilage matrix metabolism.

Also, our study used an HA product in the medium-molecular-weight range of 500,000 to 730,000 d to simulate what is widely commercially available for use in equine practice. We chose to use a medium-molecular-weight HA on the basis of results of a large analysis recently published suggesting that a medium-molecular-weight HA is the most therapeutic for intra-articular administration. However, use of a higher-molecular-weight HA in our in vitro study may have yielded different results. It could also be possible that HA may act by other mechanisms to affect matrix metabolism, such as inducing endogenous HA production that was not measured in our study. However, results of our study did indicate that HA causes no detrimental effect on cartilage PG metabolism in vitro.
Although MPA negatively affected articular cartilage matrix metabolism by decreasing PG synthesis and increasing the total release of GAG into the medium, the presence of the corticosteroid appeared to decrease the degradation and release of newly synthesized PG into the medium. This effect has been found in previous studies and reported as a protective effect of MPA on PG degradation. However, the decrease in the release of newly synthesized PG into the medium is likely a result of the MPA-induced decrease in total new PG synthesis (Table 1). The percentage of the newly synthesized PG that is released into the medium actually increases in response to increases in MPA concentration. Although it may appear that a protective effect of MPA exists on the degradation of newly synthesized PG, this is not supported when expressed as a percentage of the total newly synthesized PG. This finding also helps explain why an apparent protective effect is observed with regard to newly synthesized PG, but not in terms of the total GAG content of the medium.

Unlike the study by Dechant et al, treatment of articular cartilage explants with MPA did not decrease total explant DNA in our study. In that study, explants were treated with MPA and interleukin-1 simultaneously and cultures were performed for 8 days. Although our study was performed on articular cartilage for 3 days, exposure to MPA and HA was only for 24 hours to more closely replicate in vivo concentrations found in the joint and clearance from the joint. Had exposure to MPA been longer in our study, we may have observed a decrease in total explant DNA content. In addition, we did not use interleukin-1 in our study and interleukin-1 may have an additional detrimental effect when combined with MPA on the cartilage DNA. Also, a measurement of DNA content does not necessarily mean the cells are alive, just that the DNA is still intact. Histologic examination in conjunction with DNA analysis may be a better indicator of chondrocyte death.

Recently, because of finding discrepancies, questions were raised by Dechant et al. as to the relevance of an in vitro model to assess the effects of corticosteroids on articular cartilage. However, this type of model is needed for assessing sulfate incorporation into articular cartilage as the most sensitive test of PG synthesis and degradation. Although an in vivo model would better represent corticosteroid-induced matrix metabolism in horses, our study used 16 treatment groups, which would require a large number of horses. An in vitro model was logistically better suited to our study and negated the need to euthanatize a large number of horses.

In conclusion, findings in our study largely did not support the hypothesis that HA would mitigate MPA-induced negative effects on cartilage PG metabolism. Addition of HA at 1 mg/mL to MPA-treated cartilage may have beneficial effects on matrix metabolism, but further investigation is necessary. Studies on cartilage affected by OA or on interleukin-1–stimulated cartilage may provide stronger evidence that HA has positive matrix metabolism effects on MPA-treated cartilage. Further investigation into the effect on endogenous HA production and inflammatory mediators may also help provide insight into the pathway through which HA improves joint function.

References