Effects of methylprednisolone acetate and glucosamine on proteoglycan production by equine chondrocytes in vitro

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Objective—To evaluate the effects of methylprednisolone acetate (MPA) on proteoglycan production by equine chondrocytes and to investigate whether glucosamine hydrochloride modulates these effects at clinically relevant concentrations.

Sample Population—Articular cartilage with normal gross appearance from metacarpophalangeal and metatarsophalangeal joints of 8 horses (1 to 10 years of age).

Procedures—In vitro chondrocyte pellets were pretreated with glucosamine (0, 1, 10, and 100 µg/mL) for 48 hours and exposed to MPA (0, 0.05, and 0.5 mg/mL) for 24 hours. Pellets and media were assayed for proteoglycan production (Alcian blue precipitation) and proteoglycan content (dimethylmethylene blue assay), and pellets were assayed for DNA content.

Results—Methylprednisolone decreased production of proteoglycan by equine chondrocytes at both concentrations studied. Glucosamine protected proteoglycan production at all 3 concentrations studied.

Conclusions and Clinical Relevance—Methylprednisolone, under noninflammatory conditions present in this study, decreased production of proteoglycan by equine chondrocytes. Glucosamine had a protective effect against inhibition of proteoglycan production at all 3 concentrations studied. This suggested that glucosamine may be useful as an adjunct treatment when an intra-articular injection of a corticosteroid is indicated and that it may be efficacious at concentrations relevant to clinical use. (Am J Vet Res 2008;69:1123–1128)
can production. Additionally, an MPA-induced decrease in type II procollagen mRNA synthesis dissipated in the presence of lipopolysaccharide in cartilage of horses in vitro.\textsuperscript{13}

In several studies,\textsuperscript{6–10} the use of concurrent adjunc treatments in an effort to prevent the deleterious effects of MPA on cartilage proteoglycan homeostasis has been investigated. Intramuscular administration of polysulfated GAGs in horses fails to protect cartilage of the middle carpal joint from MPA-induced local GAG depletion.\textsuperscript{16} Sodium hyaluronate cotreatment with MPA fails to mitigate the detrimental effects of MPA alone on proteoglycan production and degradation in cartilage explants.\textsuperscript{17} In contrast, sodium hyaluronate cotreatment with MPA has beneficial effects on production of proteoglycan by equine chondrocytes in the presence of interleukin-1.\textsuperscript{16} Results of these studies\textsuperscript{6–10} indicate that although sodium hyaluronate and MPA cotreatment may have some usefulness in an inflammatory environment, the traditionally used osteoarthritis treatment adjuncts, polysulfated GAGs and sodium hyaluronate, do not alter MPA-induced proteoglycan production derangement.

The 6-carbon amino sugar glucosamine has been used as an alternative treatment for arthritis, with a growing body of evidence in support of its pain-relieving and potentially chondroprotective properties.\textsuperscript{10–22} Notably, glucosamine has beneficial effects on production of proteoglycan by chondrocytes. Glucosamine prevents degradation of cartilage proteoglycans in vitro,\textsuperscript{19,23} and it also upregulates production of proteoglycan by chondrocytes.\textsuperscript{24} Preservation of extant cartilage proteoglycans may result from the ability of glucosamine to suppress matrix metalloproteinase and aggrecanase degradative enzymes.\textsuperscript{25} Proteoglycan production may be upregulated through an increase in aggregan gene expression secondary to increases in transforming growth factor–β1 production.\textsuperscript{26}

In vitro studies\textsuperscript{19,23} investigating the effects of glucosamine on cartilage have typically used hyperphysiologic concentrations up to 25,000 μg/mL. Glucosamine, when administered at doses 10-fold higher than those used clinically, achieves blood concentrations of only 10.6 μg/mL.\textsuperscript{27} Chemically measured glucosamine concentrations in synovial fluid are < 10% of the achieved serum concentration.\textsuperscript{28} Results of recent studies\textsuperscript{25,26} support the use of glucosamine at concentrations approaching those consistent with in vivo pharmacokinetic data.\textsuperscript{27,28} Consequently, it is important to evaluate the effects of glucosamine at clinically relevant concentrations.

The objective of the study reported here was to evaluate the effect of MPA on production of proteoglycan by equine chondrocytes in vitro and to investigate whether glucosamine modulates these effects at clinically relevant concentrations. Our hypothesis was that MPA would prove detrimental to the production of proteoglycan by chondrocytes and that glucosamine would mitigate these harmful effects.

**Materials and Methods**

**Tissue sources**—Articular cartilage with gross normal appearance from metacarpophalangeal and metatarsophalangeal joints was obtained from 8 horses (1 to 10 years of age) that had died or had been euthanized with an overdose of pentobarbital for reasons other than joint disease. Cartilage was dissected from the subchondral bone and incubated at 37°C for 1 hour in physiologic saline (0.9% NaCl) solution containing 1% penicillin and streptomycin.\textsuperscript{29} Chondrocytes were isolated by 16-hour digestion at 37°C with 0.13% collagenase\textsuperscript{26} in Dulbecco modified Eagle medium.\textsuperscript{1} Following digestion, chondrocytes were counted by use of a hemacytometer and viability was determined via trypan blue-stain exclusion.\textsuperscript{2} Chondrocytes were separated by centrifugation (300 × g for 5 minutes), washed, and resuspended in Dulbecco modified Eagle medium supplemented with 1% penicillin-streptomycin, 4.5 g of glucose/L, 1% l-glutamine,\textsuperscript{30} 50 μg of ascorbic acid/mL,\textsuperscript{3} and 10% fetal bovine serum.\textsuperscript{8}

**Chondrocyte pellets**—Pellets (5 × 10\textsuperscript{6} cells) were formed by centrifugation (300 × g for 5 minutes) in 15-mL polypropylene centrifuge tubes and incubated under standard cell culture conditions (37°C in 95% relative humidity with 5% carbon dioxide). Medium was renewed every 2 to 3 days. On day 7, pellets were supplemented with glucosamine\textsuperscript{8} (0, 1, 10, and 100 μg/mL) and incubated for an additional 48 hours. At 48 hours after addition of glucosamine, media were renewed with the same concentrations of glucosamine and MPA (0, 0.05, and 0.5 mg/mL) was added. Pellets were radiolabeled with media containing sulfur 35 (\textsuperscript{35}S)–labeled sodium sulfate (10 μCi/mL).\textsuperscript{1} Twenty-four hours later, pellets and media were collected and stored at −80°C.
Proteoglycan—Radiolabeled pellets were papain digested (150 µg of papain/mL) at 65°C for 24 hours. Precipitation with Alcian blue dye and scintillation of the explant digests and media were used to determine new synthesis of proteoglycan as a result of incorporation of 35S into the cartilage matrix. Results were expressed as counts per minute per chondrocyte pellet. Total proteoglycan was calculated by adding the amount of proteoglycan produced in pellets to the amount of proteoglycan released into the media.

GAG—Media were digested in papain (7.5 mg/mL) at 65°C for 4 hours. The 1,9-dimethylmethylene blue assay was performed on digested media and pellets by use of the direct spectrophotometric method to measure the total GAG content in the spent media and explant digests. Results were compared with a chondroitin sulfate standard curve and corrected for total digestion volume (pellets) or volume in culture (media).

DNA—Total chondrocyte pellet DNA was determined from the papain digest by use of a fluorometric dye assay and a microplate reader, as previously described. Values were compared with a standard curve of calf thymus DNA and corrected for total digestion volume.

Statistical analysis—Data were compared by use of repeated-measures ANOVA with a statistical software program. Values of P < 0.05 were considered significant. Nonparametric data were compared by use of repeated-measures ANOVA on ranks. Post hoc analysis was accomplished by use of the Student-Newman-Keuls method.

Results

Proteoglycan—Treatment of chondrocyte pellets with MPA at 0.05 and 0.5 mg/mL significantly decreased proteoglycan production by 37% and 75%, respectively, compared with proteoglycan production by the negative control chondrocyte pellets (Figure 1). The combination of MPA and glucosamine at 0.5 mg/mL and 10 µg/mL, respectively, significantly increased proteoglycan production, compared with glucosamine alone at 10 µg/mL. Glucosamine did not increase baseline proteoglycan production at any concentration studied. Glucosamine significantly increased production of proteoglycan in MPA-treated chondrocyte pellets at all concentrations. Compared with production of proteoglycan in chondrocyte pellets treated with MPA at 0.05 mg/mL, treatment with glucosamine at 1, 10, and 100 µg/mL increased proteoglycan production by 33%, 58%, and 43%, respectively. Compared with production of proteoglycan in chondrocyte pellets treated with MPA at 0.5 mg/mL, treatment with glucosamine at 1, 10, and 100 µg/mL increased proteoglycan production by 366%, 550%, and 341%, respectively.

Treatment with MPA did not significantly decrease the amount of proteoglycan released into the media at either concentration studied (Figure 2). Glucosamine did not significantly increase the amount of baseline proteoglycan released into the media at any concentration studied. Glucosamine significantly increased the amount of proteoglycan released into the media of chondrocyte pellets treated with MPA at 0.05 mg/mL, treatment with glucosamine at 1, 10, and 100 µg/mL increased proteoglycan production by 246%, 175%, and 400%, respectively.

Total proteoglycan (ie, amount of proteoglycan produced in pellets plus the amount of proteoglycan released into the media)
Likewise, treatment with MPA at 0.5 mg/mL significantly increased the amount of GAG released into the media, compared with the amount of GAG released into the media of negative control chondrocyte pellets (Figure 5). Treatment with MPA at 0.5 mg/mL also significantly increased the amount of GAG released into the media of chondrocyte pellets treated with glucosamine at 1 µg/mL.

DNA—Total pellet DNA content was not significantly affected by MPA alone, glucosamine alone, or any treatment combination of MPA and glucosamine.

Discussion

Results of this study support our hypothesis that MPA is detrimental to the production of proteoglycan by equine chondrocytes in vitro and that glucosamine decreases this effect. Proteoglycan production by equine chondrocytes was dramatically depressed by MPA at concentrations of 0.05 and 0.5 mg/mL. Glucosamine significantly ameliorated this decrease in proteoglycan production at every concentration studied (1, 10, and 100 µg/mL).

Our finding that MPA decreases proteoglycan production is consistent with the results of other studies on articular cartilage under noninflammatory culture conditions. The ultimate result of these effects in vivo is a change in cartilage morphology, presumably leading to gross disruption under load, which may set permanent osteoarthritis in motion. Although the effects of MPA on articular cartilage may differ under inflammatory conditions, the cumulative data highlight what unintended clinical consequences may occur, particularly if joints are treated with high concentrations of MPA.

The ability of glucosamine to counteract an MPA-induced decrease in proteoglycan production is likely related to its ability to enhance extracellular matrix production. Results of a study indicate that glucosamine regulates the production of transforming growth factor–β1 through modulation of the hexosamine biosynthesis pathway. Glucosamine-induced upregulation of transforming growth factor–β1 is thought to be responsible for increased matrix production, including the observed upregulation of aggrecan in chondrocytes.

Notably, MPA may interfere with the production of proteoglycan by chondrocytes through downregulation of transforming growth factor–β1. Systemic administration of MPA decreases transforming growth factor–β1 production in tenocytes. We believe glucosamine may counteract the negative effects of MPA on proteoglycan production, at least in part through preservation of transforming growth factor–β1 production.

In our study, glucosamine did not increase production of proteoglycan in chondrocyte pellets in culture media) reflected the findings in chondrocyte pellets (Figure 3). Treatment with both concentrations of MPA significantly decreased total proteoglycan, compared with total proteoglycan of negative control chondrocyte pellets. Glucosamine did not increase baseline total proteoglycan at any concentration studied. All concentrations of glucosamine significantly increased total proteoglycan in MPA-treated samples, compared with treatment with MPA alone.

GAG—Treatment with MPA at 0.5 mg/mL significantly increased baseline pellet GAG content, compared with GAG content of negative control chondrocyte pellets (Figure 4). Likewise, treatment with MPA at 0.5 mg/mL significantly increased GAG content of chondrocyte pellets treated with glucosamine at 10 µg/mL or 100 µg/mL, compared with pellets not exposed to MPA. Treatment with MPA at 0.05 mg/mL significantly increased GAG content of chondrocyte pellets treated with glucosamine at 100 µg/mL.

Treatment with MPA at 0.5 mg/mL significantly increased the amount of GAG released into the media, compared with the amount of GAG released into the media of negative control chondrocyte pellets (Figure 5). Treatment with MPA at 0.5 mg/mL also significantly increased the amount of GAG released into the media of chondrocyte pellets treated with glucosamine at 1 µg/mL.

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Figure 4—Mean ± SE content of GAG in chondrocyte pellets as determined by dimethylmethylene blue assay following treatment of chondrocyte pellet cultures with GlcN (0, 1, 10, and 100 µg/mL) and MPA (0 [white bar], 0.05 [hatched gray bar], and 0.5 mg/mL [black bar]). See Figure 1 for key.

Figure 5—Mean ± SE amount of GAG released into the media as determined by dimethylmethylene blue assay following treatment of chondrocyte pellet cultures with GlcN (0, 1, 10, and 100 µg/mL) and MPA (0 [white bar], 0.05 [hatched gray bar], and 0.5 mg/mL [black bar]). See Figure 1 for key.
conditions free of MPA. The amount of proteoglycan released into the media was increased by 1.9- and 2.2-fold at glucosamine concentrations of 1 µg/mL and 10 µg/mL, respectively, although these differences were not significant. Failure to achieve significance with these data is likely the result of high variability between samples. In addition, experimental conditions in the present study may not have been optimized for enhancement of baseline proteoglycan production. Glucosamine increases production of proteoglycan by chondrocytes, although this effect is not observed at concentrations closer to those clinically achieved after oral administration. Findings of another study indicate that effects of glucosamine on chondrocyte matrix synthesis are concentration dependent and that maximal effects may be achieved within a narrow range of ideal concentration. Glucosamine had no effect on pellet GAG content or on the amount of GAG released into the media. It is likely that accumulated GAG was much greater than GAG accumulated secondary to proteoglycan production during the final 24 hours of the experiment.

One surprising and unexpected finding was the MPA-associated increase in pellet GAG content and the amount of GAG released into the media. It is not likely that this observation can be attributed solely to the reported protective effects of MPA on GAG degradation because the amount of GAG was increased in pellets and media. One potential explanation is the possibility that MPA treatment led to a decrease in extant pellet GAG degradation as well as synthesis and liberation of a population of smaller proteoglycans. These small proteoglycans may have been lost during processing for the Alcian blue precipitation assay; while remaining for the dimethylmethylene blue assay. Alternately, non-GAG species that may have been liberated interfered with dimethylmethylene blue assay results, as this phenomenon has been previously reported with exogenous hyaluronic acid supplementation of media.

Neither glucosamine nor MPA had any effect on pellet DNA content. This outcome is likely the result of the short period during which pellets were exposed to MPA. These results are consistent with the findings of another study conducted under noninflammatory conditions. In a prior study in which MPA was found to have negative effects on chondrocyte DNA content, higher concentrations of MPA, concurrent interleukin-1 treatment, and extended time points were used.

Mention should be made of the specific corticosteroid preparation used in this study. Methylprednisolone is considered to have an intermediate duration of action, and the acetate ester form is poorly water soluble, which subsequently prolongs activity. Although the acetate form renders speculation about the exact concentration of MPA in solution difficult, the use of MPA in the present study can be justified by the fact that it is the most often clinically used form of this corticosteroid. Results of a study in which water-soluble methylprednisolone sodium succinate was used indicate that the effects of methylprednisolone sodium succinate are comparable to those of MPA.

In recent studies, concentrations of glucosamine likely to be achieved after oral administration in several species have been elucidated. Achievable serum glucosamine concentrations in humans after treatment have been reported up to 2 µg/mL. Achievable serum glucosamine concentrations in horses after oral administration are between approximately 1 and 10 µg/mL, depending on the dose given. The concentrations of glucosamine used in the present study are relevant to clinically attained serum concentrations after oral administration. Particularly interesting is the observation that maximal protective effects of glucosamine on production of proteoglycan in MPA-treated chondrocytes occurred between 1 and 10 µg/mL, rather than at 100 µg/mL, although this difference was not significant. Evidence is found from the results of other studies that an optimal concentration of glucosamine may exist at which matrix synthesis is maximally enhanced and beyond which beneficial effects are gradually lost. Although the possibility has not been investigated, intra-articular administration of glucosamine would afford clinicians more precise control over the concentration within the joint.

Results of our study confirmed the negative effects of MPA on production of proteoglycan by chondrocytes and have demonstrated that glucosamine decreased these effects. The use of clinically achievable glucosamine concentrations may lend clinical relevance to our results. Although caution must be exercised when extrapolating from in vitro to in vivo systems, the evidence provided suggests that glucosamine may have use in modulating the negative effects of MPA on proteoglycan production by chondrocytes.

References