Diseases of the musculoskeletal system are the leading cause of poor performance, economic loss, and training days lost in the equine athlete. Trauma to articular cartilage, subchondral bone, and surrounding soft tissue leads to joint inflammation and, subsequently, to progressive changes of osteoarthritis. Intra-articular administration of corticosteroids, including TA, rapidly resolves joint effusion, synovitis, and pain associated with osteoarthritis and remains the mainstay of therapeutic management in equine athletes. However, considerable controversy exists over the relative benefits of intra-articular corticosteroid administration. Findings of several in vivo and in vitro studies in horses suggest that the corticosteroid methylprednisolone acetate has deleterious effects on cartilage metabolism while temporarily masking clinical signs of joint disease. Conversely, authors of several in vitro studies...
investigating different corticosteroids in chondrocyte cultures reported the absence of detrimental effects of betamethasone14 and even beneficial effects of TA,13 on articular cartilage metabolism. Potentially chondroprotective steroids, such as TA, have not been investigated in combination with other commonly administered joint medications, of which some may be detrimental to articular cartilage.

Intra-articular injection of HA is an alternative treatment for joint disease with an inflammatory component and is commonly performed simultaneously with intra-articular administration of corticosteroids.16,17 Sodium hyaluronate alone has anti-inflammatory properties that have been observed for in vitro and in vivo studies.18–22 Concentration of articular cartilage degradation products in synovial fluid can be decreased when intra-articularly administered corticosteroids are combined with HA.18,23 This finding strongly suggests that synergistic effects exist among intra-articular medications. Results of an in vitro study19 on equine articular cartilage explants revealed that addition of HA to culture media had no measurable effect on cartilage degradation induced by high doses of the corticosteroid methylprednisolone acetate.

Amikacin sulfate is an aminoglycoside antimicrobial that is commonly administered intra-articularly in horses for the treatment of septic arthritis or to prevent infection while concurrently injecting other medications.24 Medications such as TA and HA could potentially be less effective in the presence of AS but may protect chondrocytes against potential AS-induced toxic effects.

Mepivacaine hydrochloride is an amide-type sodium channel–blocking local anesthetic that is extensively used in equine practice to provide intra-articular and perineural analgesia during lameness examinations.25 Anti-inflammatory effects of a similar anesthetic, lidocaine hydrochloride, have been documented after IV administration in humans26 and horses27 but, to our knowledge, have not been documented in an articular environment. Additionally, the influence of MC on the documented anti-inflammatory efficacy of TA and HA has not been evaluated.

In equine practice, a diagnostic injection of MC may be combined with simultaneous administration of therapeutic agents, such as TA, HA, or AS, to reduce the number of joint injections and decrease associated risks. Further investigation of the effects of combining commonly used drugs on the joint environment, specifically the articular cartilage, would provide information on potentially beneficial or detrimental combinations.

The purpose of the study reported here was to evaluate the effects of TA, HA, AS, and MC, alone or in combination, on histomorphometry, histochemistry, and GAG composition of LPS-challenged and unchallenged equine articular cartilage explant cultures. Articular cartilage explants represent a valid model to assess the effects of different agents on the ability of equine chondrocytes to maintain a functional cartilage matrix.20,21,28,29 We hypothesized that LPS at a dose of 100 ng/mL would induce detrimental effects on chondrocyte health and GAG degradation and that TA and HA would be protective against, and AS and MC neutral toward, these effects.

Materials and Methods

Animals—Two adult horses (aged 11 and 13 years) that were sound at the trot with no palpable abnormalities to their femoropatellar joints were euthanized by IV administration of an overdose of pentobarbital for reasons unrelated to the musculoskeletal system. Femoropatellar joints were opened and deemed grossly normal. All procedures were approved by the Institutional Animal Care and Use Committee of The Ohio State University.

Cartilage explant cultures—By use of an aseptic technique, full-thickness cartilage was shaved from the lateral and medial trochlear ridges of the distal aspect of the femur and from the caudal surface of the patella and immediately stored in Dulbecco modified Eagle medium1 at 4°C. At the laboratory, shaved specimens were pooled, minced, and aliquoted into 12-well tissue culture plates (50 to 100 mg of wet wt/well), producing 48 wells/horse. Culture medium (Dulbecco modified Eagle medium1 supplemented with 10% fetal bovine serum, b) was added, and explants were incubated for 24 hours at 37°C in 5% carbon dioxide and 95% air. After 24 hours, the medium was changed and explants were incubated for another 24 hours in culture medium alone or with 100 mg of LPS/100 mL of medium. This process was repeated after 24 hours to provide 48 hours of LPS exposure for LPS-challenged cultures.

Treatments—After 72 hours of culture, with and without 48 hours of LPS exposure, exhausted media were removed and medium with no drug or medium with TA (0.6 mg/mL of medium), HA (2.0 mg/mL of medium), AS (25 mg/mL of medium), and MC (20 mg/mL of medium) individually or as TA plus HA, AS, or MC; HA plus AS; HA plus MC; AS plus MC; and HA plus AS plus MC (12 total groups) was added to duplicate wells of tissue culture plates. Every 24 hours, exhausted media were removed and the explants were reincubated under their individual specific treatment conditions for a total of 96 hours. Half of the cartilage explants in each well were then processed for total GAG analysis, and the other half were processed for histochemical and histomorphologic-histomorphometric evaluation.

GAG content in cartilage—Total GAG content in cartilage was assayed by use of a 1,9-dimethyl-methylene blue dye binding spectrophotometric assay.30 Briefly, cartilage specimens were incubated in acetone overnight at 4°C and subsequently dried at 37°C for 30 minutes. The dry weight of each sample was recorded, and 0.1 mL/mg of dry wt of papain digestion solution (1 mg/mL of papain in 0.69 g of dibasic sodium phosphate, 0.0326 g of 1H-1H-acetylcysteine, 0.076 g of EDTA tetrasodium salt, and nuclelease-free water to 100 mL; pH, 6.3) was added. Samples were digested at 65°C for 3 hours and diluted 1:20 for spectrophotometric analysis. Two hundred fifty microliters of dye dilution (16 mg of 1,9-dimethyl-methylene blue, 3 mL of 95% ethanol, 2 mL of formate buffer, and nuclelease-free water to 1,000 mL) was added to 50 mL of diluted sample, and optical density at 535 nm was determined on a multiple detection plate reader.6 Chondroitin sulfate A of bovine
tracheal origin\textsuperscript{6} was used to construct the standard curve. All samples were run in triplicate, and the total cartilage GAG content was reported as the mean of the 3 measurements in micrograms of GAG normalized to milligrams of tissue dry weight.

**Histologic preparation and evaluation**—Cartilage explants from each well were collected after 96 hours of treatment and fixed in 4% paraformaldehyde for 24 hours prior to immersion in PBS solution at 4°C. Samples were then dehydrated in graded concentrations of alcohol, embedded in paraffin, sectioned at 6 μm, and stained with toluidine blue for matrix GAG staining intensity and HE for chondrocyte morphology evaluation at 400× magnification. Three representative sections of the superficial, middle, and deep layers of cartilage from each well of the tissue culture plates were selected; scored from 0 to 4 (0 = very mild, 1 = mild, 2 = moderate, 3 = intense, and 4 = very intense) for intensity of toluidine blue staining; and quantified for total lacunae, empty lacunae, and lacunae containing pyknotic chondrocyte nuclei per hpf by 3 investigators (DMB, SEW, and ALB). Investigator values and results for each section were reported as the median of the 3 investigators and mean of 3 sites; findings were expressed per cartilage zone as GAG staining intensity score, percentage of empty lacunae, and percentage of lacunae containing pyknotic chondrocyte nuclei.

**Statistical analysis**—Data were analyzed by use of a statistical software program.\textsuperscript{1} Overall LPS effect on GAG content and histomorphometry was analyzed by use of repeated-measures ANOVA with 1 dummy variable for LPS treatment (ie, value = 1 if explant was challenged with LPS, and value = 0 if explant was unchallenged) as the explanatory variable. Horses were treated as a random variable, repeated measures were considered to be nested within horse, and the data in 3 zones (ie, superficial, middle, and deep) and the unchallenged and LPS-challenged groups were analyzed separately. Overall drug effects on GAG contents and histomorphometry were similarly analyzed by use of repeated-measures ANOVA with 4 dummy variables for each of TA, HA, AS, and MC treatments (ie, value = 1 if an explant received the drug, and value = 0 if otherwise) as explanatory variables. A value of $P < 0.05$ was considered significant for these analyses. Individual and combined drug effects on GAG content and histomorphometry were analyzed by use of repeated-measures ANOVA with multiple comparisons, with untreated or single drug treatment groups compared with all other combined treatment groups. By use of crude Bonferroni correction for these multiple comparisons, a value of $P < 0.005$ was considered significant for these analyses. Because of the significant overall effects of LPS on outcomes, multiple comparisons were only made within each of the unchallenged and LPS-challenged groups.

**Results**

All explants were evaluated after completion of the study, resulting in 4 wells for GAG analysis and histologic assessment for each of the 12 treatment conditions. Because of lack of statistical difference among zones of articular cartilage, histomorphometric data were combined for representation in tables and graphs.

GAG content—Treatment of equine articular cartilage explant cultures with single drugs or combinations of TA, HA, AS, and MC did not result in significant differences in total cartilage GAG content between the different treatment regimens or between LPS-challenged and unchallenged explants.

Histochemical staining intensity—Toluidine blue staining intensity increased from the superficial zone (median score, 1) through the deep zone (median score, 3), representing distribution of the GAG content in the histologic specimens. No difference was detected between unchallenged and LPS-challenged explants or among groups.

**Histologic evaluation**—Lipopolysaccharide and individual drugs significantly altered cartilage morphology, compared with groups not containing LPS or the drug, respectively (Table 1). Lipopolysaccharide exposure at 100 ng/mL increased the number of empty lacunae ($P = 0.05$) and pyknotic nuclei ($P < 0.001$) in all zones of the explants. In unchallenged explants, TA, regardless of other treatments, decreased empty lacunae in all zones ($P < 0.001$), confirming a supportive influence of TA on stressed chondrocytes in vitro. In LPS-challenged explants treated with TA, regardless of other treatment, no change was found in empty lacunae or pyknotic nuclei, compared with all other LPS groups. This indicated that TA was individually unable to fully protect explants against the detrimental effects of other treatments or LPS.

**Table 1**—Effects of LPS and drug treatments on chondrocyte histomorphometry of LPS-challenged and unchallenged equine articular cartilage explants.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Unchallenged explants (n = 48)</th>
<th>LPS-challenged explants (n = 48)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% Empty lacunae</td>
<td>% Pyknotic nuclei</td>
</tr>
<tr>
<td>LPS</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>TA</td>
<td>↓↓↓↓</td>
<td>NSD</td>
</tr>
<tr>
<td>HA</td>
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<tr>
<td>AS</td>
<td>↑↓</td>
<td>↑↑↓</td>
</tr>
<tr>
<td>MC</td>
<td>NSD</td>
<td>↑↑↑</td>
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</table>

$↑↑↑↑↑↑ = $ Significant increase ($P < 0.05$ and $P < 0.001$, respectively), compared with all other treatments within unchallenged or LPS-challenged explants. $↓↓↓↓ = $ Significant decreases ($P < 0.01$ and $P < 0.001$, respectively), compared with all other treatments within unchallenged or LPS-challenged explants. $NSD = $ Not significantly different. $NA = $ Not applicable.
In unchallenged explants, HA caused a decrease in empty lacunae ($P < 0.001$) and pyknotic nuclei ($P = 0.01$), compared with groups without HA. In LPS-challenged explants treated with HA, no change was found in empty lacunae or pyknotic nuclei, compared with all other LPS-challenged groups, indicating that HA individually, similar to TA, was unable to fully protect explants against the detrimental effects of other treatments or LPS (Table 1).

In unchallenged explants, AS caused an increase in empty lacunae ($P = 0.05$), particularly in the middle and deep layers, compared with groups without AS (Table 1). In LPS-challenged explants, treatment with AS increased pyknotic nuclei ($P < 0.001$), compared with LPS without AS.

In unchallenged explants, MC caused an increase in pyknotic nuclei ($P < 0.001$), particularly in the more...
superficial and middle zones, compared with treatments without MC (Table 1). Mepivacaine hydrochloride in combination with LPS caused an increase in empty lacunae and pyknotic nuclei ($P < 0.001$), which included the deeper cartilage zones. Mepivacaine hydrochloride amplified the negative influence of LPS on chondrocytes.

Results of multiple-comparison, posttest analyses of drug effects, in which $P < 0.005$ was considered significant, revealed that AS and MC increased empty lacunae ($P < 0.001$) and MC increased pyknotic nuclei ($P < 0.001$; Figures 1–3). This negative influence on explant morphology was attenuated by TA and HA in unchallenged explants. In LPS-challenged explants, TA and HA individually decreased empty lacunae ($P < 0.001$), TA decreased pyknotic nuclei ($P = 0.003$), and TA and HA in combination decreased empty lacunae ($P = 0.003$), compared with untreated LPS. In unchallenged explants, treatment with the combination of TA and HA did not result in any different variables, compared with explants treated independently with TA or HA. In LPS-challenged explants, the combination of MC with any treatment, even when combined with TA and HA, resulted in greater empty lacunae and pyknotic nuclei ($P < 0.001$) than LPS alone. Treatment with TA or HA, or TA in combination with HA, was unable to attenuate the pyknotic effect of LPS and MC combined.

**Discussion**

Similar to the results of previous investigations, exposure of equine articular cartilage explants to LPS at the relatively high concentration used in this study induced considerable chondrocyte injury. This was revealed by the significantly greater number of empty lacunae containing pyknotic chondrocyte nuclei in all cartilage zones. This effect permitted evaluation of both an improvement in chondrocyte morphology and a deterioration induced by the various drugs and combinations of drugs investigated.

Results of our study indicated that treatment with TA or HA, alone or in combination, had supportive effects on cartilage explants in culture as indicated by the lower number of empty lacunae (TA or HA) and pyknotic nuclei (HA) in all (TA) or most (HA) cartilage zones of unchallenged explants cultured for 7 days in standard medium. This protective effect of the combination of TA and HA persisted when explants were challenged with LPS, even though TA or HA could not completely overcome the detectable negative effect of LPS, particularly with MC added, on chondrocyte morphology in all cartilage zones. This could be explained with an additive or synergistic negative effect, particularly for explants treated with MC.

Treatment with AS alone or MC alone induced an increased percentage of empty lacunae or lacunae containing pyknotic nuclei in LPS-challenged and unchallenged explants. This is indicative of a toxic effect on chondrocytes in explant cultures. Gentamicin sulfate, but not AS, has been shown to induce a transient chemical synovitis in joints of clinically normal horses. This has been assumed to be related to a low pH as well as to a direct chemical effect. Results of our study indicate that AS can have a toxic effect on equine chondrocytes in explant cultures. Importantly, TA alone or combined with HA significantly attenuated this negative effect of AS.

Intrasynovial injection of sodium channel–blocking local anesthetics, such as MC or lidocaine hydro-
chloride, can induce a temporary synovitis in horses.\textsuperscript{45} Mepivacaine hydrochloride seems to be less irritating to the articular environment than lidocaine hydrochloride, but a previous report\textsuperscript{25} regarding the relative toxicity of the 2 compounds is controversial. Dose- and time-dependent cytotoxic effects of lidocaine hydrochloride\textsuperscript{26} and bupivacaine hydrochloride\textsuperscript{27} have previously been determined by use of flow cytometry in fresh bovine articular chondrocytes in alginate bead cultures. Authors of one of these reports concluded that the observed decrease in chondrocyte viability following exposure to lidocaine hydrochloride was not mediated by a reduction in pH.\textsuperscript{28} Our study revealed potential for MC to exert toxic effects on chondrocytes, and MC was the most toxic of all the drugs that were evaluated. In contrast, lidocaine hydrochloride, administered IV as a prokinetic drug after abdominal surgery in humans\textsuperscript{29} and horses,\textsuperscript{27,28} may have anti-inflammatory effects via inhibition of local prostaglandin synthesis.\textsuperscript{20} To our knowledge, anti-inflammatory properties of MC in joints have not been described, but our histologic findings suggest that MC can, when direct contact is sustained, exert a toxic effect on chondrocytes. This negative effect was aggravated by the presence of the proinflammatory agent LPS. Concurrent use of TA and HA with MC resulted in significant suppression of this negative effect. It remains unknown whether simultaneous use of HA, TA, or both could prevent or reduce potential negative effects of proinflammatory agents on articular cartilage in vivo; however, TA or HA could not sufficiently block the toxic effects of MC in our study.

Unchallenged explants treated with the combination of TA or HA with AS had better chondrocyte morphology than did explants treated with AS alone or TA and AS without HA, showing a benefit to combining the drugs. Both TA and HA appear to have the potential to reverse or block some of the combined detrimental effects exerted by LPS and AS. A similar attenuating effect of TA, but only when combined with HA, was observed in LPS-challenged explants. This beneficial effect was no longer significant when the TA and HA combination was administered simultaneously with MC in LPS-challenged explants. These observations suggest a protective effect of TA or HA against toxic effects in articular cartilage.

Intra-articular administration of corticosteroids and other drugs remains the mainstay of therapeutic management of osteoarthritis in horses.\textsuperscript{46} In agreement with previous reports,\textsuperscript{4,15} TA at the concentration used in the present study appeared to exert a supportive effect on equine chondrocytes in explant cultures. Our findings further suggest that a combination of TA with toxic compounds, such as LPS, AS, or MC, can make this protective effect apparent, but may possibly be insufficient to maintain normal cartilage morphology. Importantly, the conditions of this static explant culture system represent a harsh environment with constant exposure of cartilage explants to all drugs. In the joint of a live horse, drugs would be cleared and buffering mechanisms would be active. Results of our study do reveal, however, the potential for these drugs to have protective (TA or HA) or toxic effects (AS or MC) on articular cartilage.

A difference in cartilage GAG content or GAG staining was not detected between different treatments or between LPS-challenged or unchallenged explants. These findings are consistent with the results of a study by Fortier et al.,\textsuperscript{39} in which alterations in GAG content were also not detected in equine synovioocyte-cartilage coculture systems exposed to matrix metalloproteinase-13 or interleukin-1α. In our study, evaluation of culture media for GAG content or inflammatory mediators, such as prostaglandin E₂, would have been an additional technique to evaluate cartilage degradation. However, on the basis of the GAG content measured within the remaining cartilage at the end of the study, at least GAG release into media may have been undetectable and unimportant in our study.

In conclusion, TA and HA appeared to exert a supportive effect on equine chondrocytes in LPS-challenged and unchallenged explant cultures in our study. Amikacin sulfate and MC exerted a toxic effect in unchallenged and LPS-challenged explant cultures, which was partially ameliorated by TA or HA. Combining TA and HA provided additional benefit in the presence of LPS challenge. Live animal studies would be necessary to determine the clinical impact of these findings in vivo and to provide the equine practitioner with applicable recommendations.

References


