Effects of hyaluronan alone or in combination with chondroitin sulfate and *N*-acetyl-D-glucosamine on lipopolysaccharide challenge-exposed equine fibroblast-like synovial cells

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OBJECTIVE
To investigate effects of hyaluronic acid (HA) or HA combined with chondroitin sulfate (CS) and *N*-acetyl-D-glucosamine (NAG) by use of a lipopolysaccharide (LPS) in vitro method.

SAMPLE
Monolayer cultures of synovial cells from 4 adult horses.

PROCEDURES
Synovial cell cultures were untreated or treated with HA alone or HA-CS-NAG for 24 hours, subsequently unchallenged or challenge-exposed with 2 LPS concentrations (20 and 50 ng/mL) for 2 hours, and retreated with HA or HA-CS-NAG for another 24 hours. Cellular morphology of cultures was evaluated at 0, 24 (before LPS), 26 (after LPS), and 50 (24 hours after end of LPS) hours. At 50 hours, cell number and viability and prostaglandin (PG) E_2, interleukin (IL)-6, matrix metalloproteinase (MMP)-3, and cyclooxygenase (COX)-2 production were measured.

RESULTS
LPS challenge exposure induced a significant loss of characteristic synovial cell morphology, decrease in cell viability, and increases in concentrations of PGE_2, IL-6, MMP-3, and COX-2. Cells treated with HA or HA-CS-NAG had significantly better viability and morphology scores and lower concentrations of PGE_2, MMP-3, IL-6, and COX-2 than untreated LPS challenge-exposed cells. Cells treated with HA had significantly better morphology scores at 50 hours than cells treated with HA-CS-NAG. Cells treated with HA-CS-NAG had significantly superior suppression of LPS-induced production of PGE_2, IL-6, and MMP-3 than cells treated with HA alone.

CONCLUSIONS AND CLINICAL RELEVANCE
HA and HA-CS-NAG protected synovial cells from the effects of LPS. Treatment with HA-CS-NAG had the greatest anti-inflammatory effect. These results supported the protective potential of HA and HA-CS-NAG treatments. (*Am J Vet Res* 2017;78:579–588)

Osteoarthritis is characterized by cellular damage and extracellular matrix degradation, which often is initiated by trauma or inflammation.[1–4] Once this process is activated, an imbalance toward a proinflammatory cascade becomes progressive, which leads to inflammation and functional joint derangements.[3,5–7] Components of synovial fluid provide lubrication and viscoelasticity to reduce wear and degradation of joints and maintain joint homeostasis.[4,8] Alterations in the concentration and molecular weight (composed of repeating disaccharides of D-glucuronic acid and NAG) of HA in synovial fluid, and a subsequent reduction in volume and viscosity of the synovial fluid, greatly diminish joint viscoelasticity.[2,9] Synovial fluid dilution, enzymatic cleavage, and altered synthesis of HA and GAGs (eg, CS-4 and CS-6) lead to altered synovial and cartilage extracellular matrix composition, decreased osmotic barrier function of the synovium, poor aggrecan quality, or HA binding that results in a severe decrease in the compression-resilient properties of cartilage.[10,11]

In addition to structural alterations, extracellular matrix cell interactions in the synovium, cartilage, and subchondral bone are altered by changes in the binding of HA, collagen, and CS to cell membrane re-

ABBREVIATIONS

COX	Cyclooxygenase
CS	Chondroitin sulfate
DMEM	Dulbecco modified Eagle medium
GAG	Glycosaminoglycan
HA	Hyaluronic acid
IL	Interleukin
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
NAG	*N*-acetyl-D-glucosamine
PG	Prostaglandin
PSGAG	Polysulfated glycosaminoglycan

ceptors (eg, CD44, toll-like receptors, and intercellular adhesion molecule 1) and modulation of transcription factors.[12–14] Activation of these cell membrane receptors via pathogen-associated molecular patterns that activate innate immune responses, extracellular matrix fragments, and cytokines can regulate inflammation and immunity during tissue repair, including regulation of immune cells such as macrophages. Targeting genes of inflammatory and catabolic mediators (eg, nitric oxide synthase-2, COX-2, and MMPs) and PGE_2 has been a major area of research for the determination of clinical sign–modifying drugs and multimodal treatment interventions for osteoarthritis.[9,15,16] Common medications used to mitigate clinical signs are NSAIDs, corticosteroids, PSGAGs, HA, and NAG.[3,17–20] When used individually, treatments have been found to have disease-modifying effects; however, controlled experimental and clinical evaluation of combinations of drugs with different mechanisms of action in an effort to target various pathways of osteoarthritis to enhance efficacy or provide synergy in the treatment of disease has not been well evaluated.[9,19]

In another study[21] conducted by our laboratory group, it was reported that HA mitigates proinflammatory effects of LPS and has anti-inflammatory and anticatabolic protective effects. Similarly, other studies[9,22,23] with HA have provided analogous results in the attenuation of inflammation through competitive inhibition and by other mechanisms, including inhibition of degradative enzymes for collagen, aggrecan, and proteoglycan by CS and upregulation of proteoglycans, GAGs, transforming growth factor-β1, and collagen type II. Furthermore, NAG has anti-inflammatory and chondroprotective effects in horses with experimentally induced osteoarthritis and, in some cases has better disease-modifying activity than HA.[19]

Although combination treatments have been used, few studies have been conducted to evaluate the actions of combination regimens or to compare effects of the components (eg, HA-CS-NAG). Intra-articular injection of HA and corticosteroids is frequently performed and has been found to decrease concentrations of articular cartilage degradation products in synovial fluid, which suggests synergetic effects.[22] In a more recent study,[24] investigators evaluated administration of a combination formulation (HA, NAG, and pentosan polysulfate) or saline (0.9% NaCl) solution administered IV to horses with an experimentally induced osteochondral fragment and found superior results in radiographic, gross cartilage, and lameness findings for the treatment group, compared with results for the placebo. Intravenous administration of HA alone was not performed in that study. Intra-articular administration of HA or PSGAG (each combined with amikacin), compared with intra-articular administration of saline solution and amikacin, to horses with an experimentally induced osteochondral fragment resulted in improvements of some synovial membrane histologic variables for HA-amikacin and improvements of cartilage fibrillation and joint effusion for PSGAG-amikacin.[19]

Synovial fibroblast cultures represent a method for assessing effects of various agents on the ability of equine synovial cells to maintain a functional extracellular synovial environment. Mediators of synovial origin have been increasingly recognized as an important part of the pathophysiologic process in osteoarthritis. Lipopolysaccharide, an element of the outer membrane of Gram-negative bacteria, can induce characteristic inflammatory processes and degradation cascades, such as increased production of PGE_2, Il-6, and MMP-3.[4] As such, use of LPS has become an established method (both in vitro and in vivo) for induction of localized inflammation and cartilage turnover.[25–27]

The objective of the study reported here was to evaluate the protective and anti-inflammatory effects of treatment with HA alone or a combination of HA-CS-NAG by use of LPS challenge exposure of synovial cell cultures. Our hypothesis was that effects of HA would differ from effects for a combination of antiarthritic components (HA-CS-NAG) with regard to cellular protection and anti-inflammatory profiles induced by LPS challenge exposure. It was anticipated that HA would provide a protective effect and that HA-CS-NAG would potentially provide an additive or synergistic protective and anti-inflammatory effect. Outcome assessments included cellular morphology scores, cell counts, and viability counts as well as measurement of PGE_2, IL-6, COX-2, and MMP-3 concentrations in media. Our goal was to define potentially beneficial effects of a combination treatment.

Materials and Methods

Sample

Tissue specimens from 4 adult horses (1 Thoroughbred, 1 Rocky Mountain Horse, and 2 Quarter Horses) that had been euthanized for reasons unrelated to the stifle joint were obtained for use in the study. The study protocol was approved by The Ohio State University Institutional Animal Care and Use Committee.

Horses were euthanized by IV administration of an overdose of sodium pentobarbital solution.[a] Within 5 hours after horses were euthanized, the stifle joints (femoropatellar and femorotibial joints) of each horse were aseptically prepared and incised and were deemed to have no abnormalities during gross examination. Specifically, macroscopic evaluation revealed no signs of inflammation; articular cartilage had no evidence of erosive lesions; periarticular osteophytes were not observed; and there appeared to be a typical quantity, viscosity, and color of synovial fluid. All villus synovium was dissected from underlying fat and adventitia, washed, and placed in DMEM[b] for transport to our laboratory.

Cell culture

Harvested synovium was rinsed with PBS solution, transferred to supplemented DMEM (supple-

mented with 10% fetal bovine serum[c] and a solution[d] containing 1% L-glutamine [29.2 mg/mL], 50 U of penicillin/mL, and 50 U of streptomycin/mL), and immediately digested with 0.02% collagenase[e] while being gently stirred for 5 hours at 37°C. Isolated cells were collected by centrifugation (500 × *g* for 5 minutes), washed, strained through a nylon-mesh strainer,[f] and seeded in supplemented growth medium in 25-cm^3 flasks.[g]

Cells were incubated for 3 days, and nonadherent cells then were removed by changing the medium. Flasks were maintained by changing medium at 48- to 72-hour intervals until cell replication at > 75% confluence was observed; at that time, synoviocytes were placed into 75-cm^3 flasks[g] and expanded in culture. Cultures were limited to passages 3 through 6 so that fibroblast-like synovial cells were the dominant cell population in the cultures; no inflammatory cells were present. Synovial fibroblast-like cells that adhered to the bottom of the 75-cm^3 cell culture flasks for each horse were compartmentalized into 4 square quadrants (18.75 cm^2) that comprised a mean of 2.0 × 10^6 cells to 2.5 × 10^6 cells in each quadrant. All flasks had similar cell populations and densities at the start of the study.

Experimental design

Monolayer cultures of equine synovial cells were used to investigate effects of 2 formulations on mediators of inflammation in response to challenge exposure with LPS at 2 concentrations (20 and 50 ng/mL). Products that contained HA[h] and a combination of HA-CS-NAG[i] were used. To elucidate potentially synergistic effects, the HA product[h] and combination HA-CS-NAG product[i] included the same HA. In addition, the HA selected was compatible with common cell-supporting media, cell surfactants, and cryopreservative solutions. Treatment groups evaluated consisted of cell cultures treated by incubation with HA or HA-CS-NAG and then challenge exposed with LPS, untreated cell cultures challenge exposed with LPS (positive control sample), and untreated cell cultures not challenge exposed with LPS (negative control sample)

Monolayer cultures for each horse were used. Medium in the 75-cm^3 flasks was changed, and fibroblast-like synovial cells at passages 3 through 6 (> 75% confluency) were incubated with HA or HA-CS-NAG. Treatments (3 mL of HA[h] [15 mg of hyaluronic sodium salt] or 3 mL of HA-CS-NAG[i] [15 mg of hyaluronic sodium salt, 100 mg of sodium CS, and 100 mg of NAG) were added to flasks that contained cells and 12 mL of supplemented DMEM (time 0). Cultures contained equal concentrations of HA that represented a single dose recommended by the manufacturer.[20,21]

LPS challenge exposure

After cells were incubated for 24 hours, medium was removed. Cells were not challenge exposed or were challenge exposed with LPS[j] (3 mL at a concentration of 20 ng/mL or 50 ng/mL). Flasks were incubated with LPS for 2 hours. At the end of the 2-hour LPS incubation (ie, 26 hours), cells were washed 3 times with a balanced salt solution,[k] and medium and treatment (3 mL of HA or HA-CS-NAG) were replaced. Cells then were incubated for an additional 24 hours until the termination of the study at 50 hours.

Cellular morphology and cell counts

Flasks were evaluated microscopically (100× magnification) for each horse, each treatment, and each LPS concentration at 0, 24, 26 (immediately after LPS challenge exposure), and 50 (24 hours after LPS challenge exposure) hours. At each time point, flask quadrants were photographed and subsequent microscopic images stored as described elsewhere.[21]

Morphology scores were assigned by use of an ordinal scale modified from another study.[21] Scores were assigned as follows: 0, > 95% attached; 1, 5% to 25% rounded or rounded and detached; 2, 26% to 50% rounded or rounded and detached; 3, 51% to 75% rounded or rounded and detached; 4, > 76% rounded or rounded and detached; 5, 5% to 25% crenated, pyknotic, and detached; 6, 26% to 50% crenated, pyknotic, and detached; 7, 51% to 75% crenated, pyknotic, and detached; and 8, > 76% crenated, pyknotic, and detached. Cells with a score of 0 to 4 included detached but viable-appearing cells, and cells with a score of 5 to 8 were detached, crenated, and pyknotic cells that were considered dead. Scores were assigned by 2 investigators (AHK and ALB) who were not aware of cell treatments. A mean value for the 2 investigators was calculated to provide a score for each assessment.

Media collection and cell harvest

At 50 hours, media were collected and frozen at –20°C for analyses of mediator concentrations. Cells were scraped from the flasks and resuspended in remaining media; total cell number was counted by use of a hemacytometer. Cells were stained with trypan blue stain.[l] Nonviable cells were counted, and that number was subtracted from the total cell number to yield the total viable cell count and the percentage of viable cells. Cell lysate was obtained by 3 rapid freeze-thaw cycles of an aliquot of cells, which was washed and suspended in lysate buffer. Lysates were frozen and stored until immediately prior to analysis.

Analysis of mediator concentrations

Media for the various treatments and times were thawed immediately before assay and maintained on ice. Concentrations of PGE-2, IL-6, and MMP-3 in cell culture media and COX-2 in cell lysate solution were determined by use of commercial competitive ELISAs[m] as described in another study[21]; ELISAs were used in accordance with manufacturer protocols. The optical density of each sample was measured by use of a microplate reader[n] and expressed as a concentration (picograms per milliliter or nanograms per milliliter). All samples were assayed in triplicate, and the mean value was calculated and used for analysis.

Statistical analysis

On the basis of data from another study[21] conducted by our laboratory group, an a priori power estimation ($\alpha = 0.05$; power = 0.8) with mean ± SEM values for PGE_2, IL-6, and MMP-3 revealed that sample size for an in vitro culture method would require 3 horses/treatment group. Thus, we anticipated that the present study had sufficient power. Data were analyzed by use of a statistical software package.[o] Significance was set at $P < 0.05$.

Objective (cell counts, cell viability, and ELISA data at the end of the study) and ordinal score data (morphology) at each time point (0, 24, 26, and 50 hours) for each treatment and each LPS concentration were assessed for normality by use of the D'Agostino and Pearson omnibus normality test. Objective data were analyzed by use of an ANOVA and a Dunn post hoc test as well as a Friedman test for multiple comparisons. For ordinal score data, a nonparametric Kruskal-Wallis analysis among treatment groups was performed, which was followed by a Wilcoxon paired rank test. Data for the ELISAs were analyzed as a concentration normalized (log base e) and tabulated as a concentration and graphed as a ratio of the concentration for the untreated unchallenged control samples to detect effects without multiple comparisons.

Comparisons among treatment groups were made by use of a Friedman test for multiple comparisons as well as by use of a nonparametric Kruskal-Wallis analysis followed by a Wilcoxon paired rank test to detect differences among treatment groups. All data were reported as mean ± SEM. All graphs were created with commercial graphing software.[o]

Results

Experimental Design

All data were collected in accordance with the study design. Synovium from each horse was cultured to a cell suspension (3 to 6 passages), with proliferating fibroblast-like synovial cells the dominant cell type in the monolayer cultures for each of the treatment groups and 2 LPS concentrations.

Cellular morphology

All evaluations were performed at 100X magnification; cells were clearly visible as discrete cells, with > 100 cells/field of view. All flasks for all treatment groups were scored as 0 or 1 at the start of the study (0 hours) and immediately before LPS challenge exposure (24 hours), including cells treated with HA or HA-CS-NAG. Cells were flat, aligned, elongated, spindle-shaped cells with nuclei (round to oval at > 75% confluence),[10] which is typical of healthy monolayer fibroblast-like synovial cells.[21]

Challenge exposure with LPS at 20 ng/mL—Positive control cells (not treated with HA or HA-CS-NAG but challenge exposed with LPS [20 ng/mL]) had significantly ($P < 0.001$) higher morphology scores than did negative control cells (not treated with HA or HA-CS-NAG and not challenge exposed with LPS) at 26 and 50 hours **(Table 1)**. Mean score for the negative control cells was 0 for the duration of the study. Morphological changes at 26 hours (ie, 2 hours after start of LPS challenge exposure) represented a loss of cell attachment to the culture flask and cell contraction and rounding. At 26 hours, cells treated with HA and HA-CS-NAG had significantly ($P < 0.001$) lower morphology scores than did the positive control cells, and cells treated with HA had significantly ($P = 0.04$) lower morphology scores than did cells treated with HA-CS-NAG. At 26 and 50 hours, HA-treated cells had recovered from LPS challenge exposure, and results for those cells did not differ significantly ($P = 0.13$) from results for the negative control cells. At 50 hours, most of the positive control cells were pyknotic. A large portion of the cells treated with HA and HA-CS-NAG had reattached and appeared elongated and spindle shaped with robust nuclei, and they were described as viable cells. Morphology scores for cells

Table 1—Variables for equine fibroblast-like synovial cells treated or not treated by incubation with HA or HA-CS-NAG and challenge exposed or not challenged exposed with LPS at 20 or 50 ng/mL.

		24 hours				26 hours				50 hours			
LPS (ng/mL)	Variable*	Control	LPS	LPS + HA	LPS + HA-CS-NAG	Control	LPS	LPS + HA	LPS + HA-CS-NAG	Control	LPS	LPS + HA	LPS + HA-CS-NAG
20	Cellular morphology	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)[a]	7.5 (6–8)[b]	2 (1–4)[c]	4 (2–6)[d]	0 (0–0)[a]	7 (5–9)[b]	0 (0–3)[a]	0.5 (0–3)[c]
20	Total cell count (X 10^4)	20	20	20	20	—	—	—	—	440 ± 63.4	1,375 ± 40.0	534 ± 129.3	963± 238.4
20	Cell viability (%)	—	—	—	—	—	—	—	—	90.0 ± 1.8[a]	60.4 ± 8.3[b]	87.0 ± 5.5[a]	68.5 ± 5.8[a,b]
50	Cellular morphology	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–1)[a]	6.5 (4–8)[b]	2 (0–4)[c]	2 (1–4)[c]	0 7. (0–0)[a]	5 (6–8)[b]	1 (1–2)[c]	1.5 (1–5)[d]
50	Total cell count (X 10^4)	20	20	20	20	—	—	—	—	599 ± 294	935 ± 375	1,830 ± 751	1,823 ± 583
50	Cell viability (%)	—	—	—	—	—	—	—	—	89.0 ± 1.5[a]	45.4 ± 15.3[b]	81.3 ± 3.4[a]	71.8 ± 3.5[c]

Cells were cultured (time 0) and treated by incubation with HA or HA-CS-NAG for 24 hours, challenge exposed with LPS (20 or 50 ng/mL) for 2 hours, and then incubated with HA or HA-CS-NAG for an additional 24 hours. Negative control cells (control) were not treated with HA or HA-CS-NAG and not challenge exposed with LPS, and positive control cells (LPS) were not treated with HA or HA-CS-NAG but were challenge exposed with LPS.

[a–d]Within a row within a time frame, values with different superscript letters differ significantly ($P < 0.05$).

treated with HA and HA-CS-NAG were significantly ($P < 0.001$) lower than the morphology scores for the positive control cells.

Statistical evaluation of treatment groups (regardless of time) revealed that the negative control cells consistently had lower morphology scores (median score, 0; which indicated a healthy cell population), compared with the morphology scores for the positive control cells (median score, 7). Cells treated with HA and HA-CS-NAG consistently had lower morphology scores than did the positive control cells, which indicated that treatment with HA or HA-CS-NAG before and after LPS challenge exposure significantly ($P < 0.001$ and $P = 0.02$, respectively) reduced the morphology scores (ie, resulted in an improvement in morphology), compared with morphology scores for the positive control cells (Table 1; **Figure 1**).

Challenge exposure with LPS at 50 ng/mL—Positive control cells (not treated with HA or HA-CS-NAG but challenge exposed with LPS [50 ng/mL]) had significantly ($P < 0.001$) higher morphology scores than did negative control cells (not treated with HA or HA-CS-NAG and not challenge exposed with LPS) at 26 and 50 hours (Table 1). Morphological changes included reversible and irreversible loss of cell attachment to the culture flask, cell contraction, and cell death evident as small, round, dark, pyknotic floating cells and cellular debris, which reflected toxicosis and cell death and was subjectively worse than for challenge exposure with LPS at 20 ng/mL (Figure 1). At 26 hours, positive control cells, cells treated with HA, and cells treated with HA-CS-NAG had significantly ($P < 0.001$, $P = 0.008$, and $P < 0.001$, respectively) higher morphology scores than did negative control cells (not treated with HA or HA-CS-NAG and not challenge exposed with LPS), and the positive control group had significantly higher morphology scores than did cells treated with HA ($P < 0.001$) or HA-CS-NAG ($P = 0.005$). At 50 hours, positive control cells continued to have significantly ($P < 0.001$) higher morphology scores than did cells treated with HA or HA-CS-NAG, and cells treated with HA had significantly ($P = 0.02$) lower morphology scores than did cells treated with HA-CS-NAG.

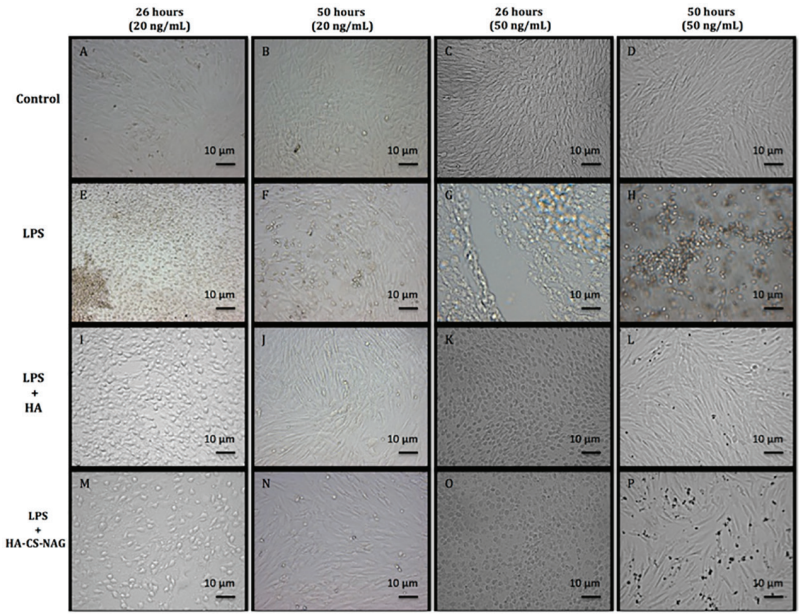

Figure 1—Representative photomicrographs of equine fibroblast-like synovial cells. Cells were cultured (time 0) and treated by incubation with HA or HA-CS-NAG for 24 hours, challenge exposed with LPS (20 or 50 ng/mL) for 2 hours, and then incubated with HA or HA-CS-NAG for an additional 24 hours. Negative control cells (control) were not treated with HA or HA-CS-NAG and not challenge exposed with LPS, and positive control cells (LPS) were not treated with HA or HA-CS-NAG but were challenge exposed with LPS. Notice that at 26 hours (2 hours after start of LPS challenge exposure) and 50 hours (24 hours after LPS challenge exposure), negative control cells had a typical morphological appearance of dense spindle-shaped cells grown to confluence and high viability (A through D), whereas positive control cells had lifting of cells at 2 hours (E) and death of cells at 24 hours (F) after LPS challenge exposure at 20 ng/mL and had lifting of sheets of cells at 2 hours (G) and death of cells at 24 hours (H) after LPS challenge exposure at 50 ng/mL. Notice that cell death in positive control cells was more pronounced for LPS challenge exposure at 50 ng/mL (viability, 42%) than at 20 ng/mL (viability, 60%). Cells treated by incubation with HA (I through L) or HA-CS-NAG (M through P) were protected from morphological changes induced by LPS (rounding, cell contraction, vacuolation, and irreversible loss of cell attachment) and had cell recovery by 50 hours, with cells reattaching or dividing with spindle-shaped morphology. Trypan blue stain; bar = 10 µm.

Statistical evaluation of treatment groups (regardless of time) revealed that the negative control cells consistently had significantly ($P < 0.001$) lower morphology scores (median score, 0; which indicated a healthy cell population), compared with morphology scores for the positive control cells (median score, 7). Treatment with HA or HA-CS-NAG before and after LPS challenge exposure significantly ($P < 0.001$) reduced the morphology scores (ie, resulted in an improvement in morphology), compared with the positive control group (Table 1; Figure 1). There was no significant difference in morphology scores between cells treated with HA or HA-CS-NAG when time was not considered as a factor, which indicated that there was a detectable delay in protection of cells incubated with HA-CS-NAG.

Cellular viability and cell counts

Total cell count (live and dead cells) increased (≥ 20-fold) from time 0 for all treatments; there was no difference among groups (Table 1; Figure 1). Percentage of cell viability was significantly ($P < 0.001$) lowest for the untreated cells challenged exposed with LPS. For LPS challenge exposure at 50 ng/mL, viable cell count differed

Table 2—Mean ± SEM concentration of mediators in media of cultured equine fibroblast-like synovial cells treated or not treated by incubation with HA or HA-CS-NAG and challenge exposed or not challenged exposed with LPS at 20 or 50 ng/mL.

	LPS at 20 ng/mL				LPS at 50 ng/mL			
Variable	Control	LPS	LPS + HA	LPS + HA-CS-NAG	Control	LPS	LPS + HA	LPS + HA-CS-NAG
PGE_2 (pg/mL)	827.8 ± 39.5[a]	6,448.6 ± 273.9[b]	713.6 ± 87.8[c]	489.0 ± 49.2[d]	1,220.4 ± 243.9[a]	1,1177.3 ± 2,430.9[b]	976.1 ± 58.8[c]	512.1 ± 100.4[d]
IL-6 (pg/mL)	15.2 ± 2.7[a]	58.7 ± 6.1[b]	35.3 ± 4.1[c]	21.7 ± 4.8[a]	26.5 ± 1.3[a]	78.8 ± 9.0[b]	37.9 ± 1.6[c]	30.3 ± 2.5[a]
MMP-3 (ng/mL)	0.82 ± 0.11[a]	2.82 ± 0.54[b]	1.36 ± 0.1[c]	0.84 ± 0.11[a]	1.43 ± 0.04[a]	2.46 ± 0.06[b]	1.64 ± 0.09[a]	1.15 ± 0.01[c]
COX-2 (pg/mL)	582.5 ± 84.8[a]	697.7 ± 69.1[b]	631.4 ± 77.7[c]	644.7 ± 76.3[d]	455.1 ± 3.3[a]	579.4 ± 21.0[b]	497.3 ± 11.6[c]	467.0 ± 10.8[a,c]

[a–d]Within a row within an LPS concentration, values with different superscript letters differ significantly ($P < 0.05$).
See Table 1 for remainder of key.

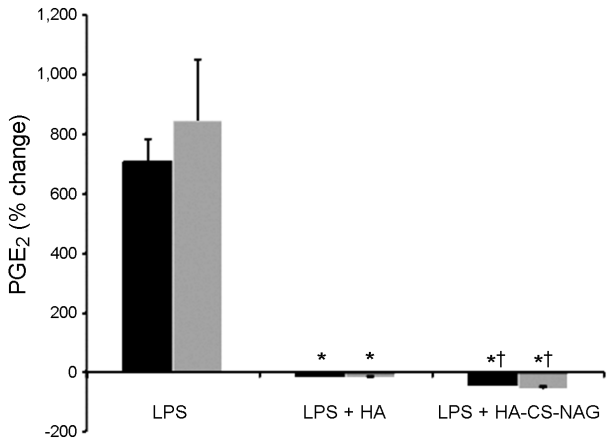

Figure 2—Mean ± SEM concentration of PGE_2 (expressed as the mean percentage change from the value for the negative control culture) for cultured equine synovial cells treated by incubation with HA or HA-CS-NAG and challenge exposed with LPS at 20 ng/mL (black bars) or 50 ng/mL (gray bars). *Within an LPS concentration, value differs significantly ($P = 0.04$) from the value for the positive control group (LPS). †Within an LPS concentration, value differs significantly ($P < 0.001$) from the value for the cells treated with HA and challenge exposed with LPS.

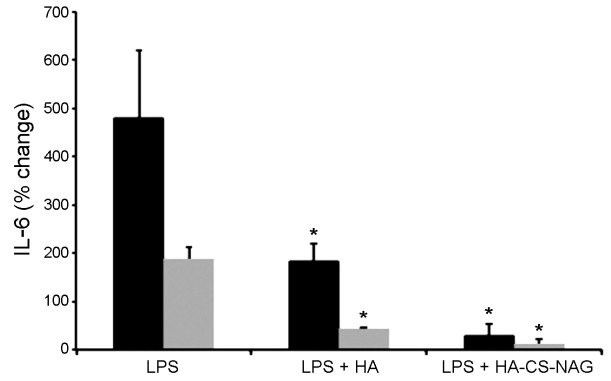

Figure 3—Mean ± SEM concentrations of IL-6 (expressed as the mean percentage change from the negative control culture) for cultured equine synovial cells treated by incubation with HA or HA-CS-NAG and challenge exposed with LPS at 20 ng/mL (black bars) or 50 ng/mL (gray bars). *Within an LPS concentration, value differs significantly ($P < 0.001$) from the value for the positive control group (LPS).

such that the positive control cells had a significantly lower viable cell count, compared with the viable cell count for the negative control cells (P

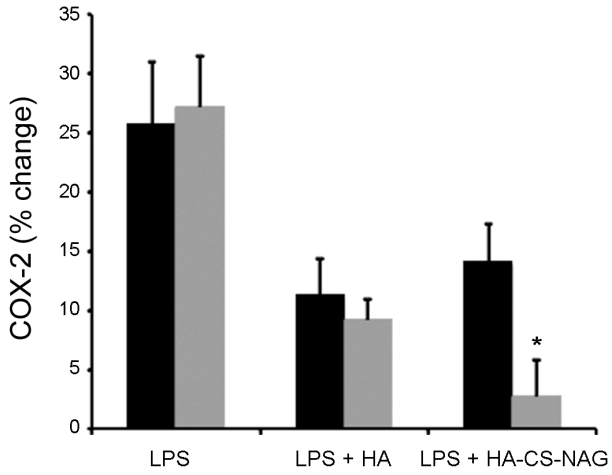

Figure 4—Mean ± SEM concentration of COX-2 (expressed as the mean percentage change from the value for the negative control culture) for cultured equine synovial cells treated by incubation with HA or HA-CS-NAG and challenge exposed with LPS at 20 ng/mL (black bars) or 50 ng/mL (gray bars). *Value differs significantly ($P < 0.001$) from the value for the positive control group (LPS) challenge exposed with LPS at 50 ng/mL.

= 0.02), cells treated with HA ($P = 0.04$), and cells treated with HA-CS-NAG ($P = 0.04$). These results indicated that LPS at 50 ng/mL killed a significant number of cells and that treatment with HA or HA-CS-NAG protected against this effect.

PGE_2, IL-6, MMP-3, and COX-2 concentrations

Challenge exposure with LPS induced significant increases in concentrations of PGE_2 (9-fold increase; $P = 0.004$), IL-6 (4-fold increase; $P < 0.001$), COX-2 (1.25-fold increase; $P < 0.001$), and MMP-3 (50 ng/mL, 1.75-fold increase [$P = 0.006$]; 20 ng/mL, 3-fold increase [$P = 0.006$]), compared with concentrations in negative control cells. Treatment with HA and HA-CS-NAG abolished the LPS effect for PGE_2 and resulted in concentrations significantly ($P = 0.04$) less than the concentration for negative control cells; in addition, cells treated with HA-CS-NAG had the lowest PGE_2 concentration, which was significantly ($P < 0.001$) lower than the PGE_2 concentration for cells treated with HA **(Table 2; Figure 2)**. Treatment with HA and

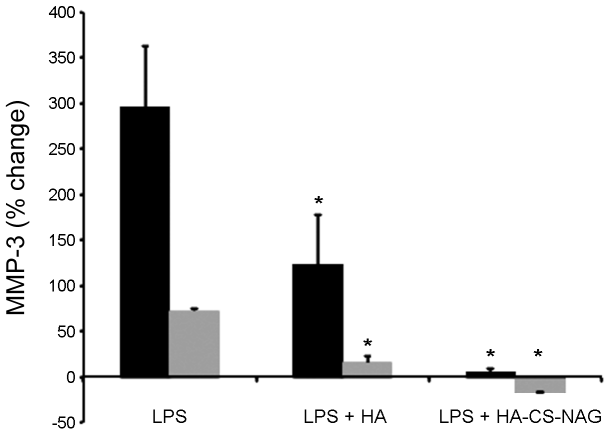

Figure 5—Mean ± SEM concentration of MMP-3 (expressed as the mean percentage change from the value for the negative control culture) for cultured equine synovial cells treated by incubation with HA or HA-CS-NAG and challenge exposed with LPS at 20 ng/mL (black bars) or 50 ng/mL (gray bars). *Within an LPS concentration, value differs significantly ($P < 0.001$) from the value for the positive control group (LPS).

HA-CS-NAG significantly ($P = 0.003$) suppressed the LPS effect for IL-6, compared with the concentration for the negative control cells, and cells treated with HA-CS-NAG had the greatest suppression of IL-6 as a result of LPS challenge exposure, with an IL-6 concentration significantly ($P < 0.001$) lower than the IL-6 concentration for HA-treated cells but not significantly different from the IL-6 concentration for the negative control cells **(Figure 3)**. Treatment with HA and HA-CS-NAG significantly ($P = 0.003$) suppressed the LPS effect for COX-2, compared with the concentration for the positive control cells **(Figure 4)**; cells treated with HA had significantly ($P = 0.002$) greater COX-2 suppression with LPS challenge exposure at 20 ng/mL, and cells treated with HA-CS-NAG had significantly ($P = 0.002$) greater COX-2 suppression with LPS challenge exposure at 50 ng/mL but did not differ significantly from results for the HA-treated cells ($P = 0.211$) or negative control cells ($P = 0.416$). Challenge exposure with LPS significantly ($P = 0.006$) increased MMP-3 concentrations **(Figure 5)**. Compared with MMP-3 concentrations for the negative control cells, cells treated with HA-CS-NAG had MMP-3 concentrations that were not significantly different when cells were challenge exposed with LPS at 20 ng/mL ($P = 0.69$) but that were significantly less when cells were challenge exposed with LPS at 50 ng/mL ($P < 0.001$). This effect was not detected for cells treated with HA. Therefore, cells treated with HA-CS-NAG had a significantly lower MMP-3 concentration than did cells treated with HA. Measurement of COX-2, expressed as a percentage change from the value for the unchallenged negative control cells, indicated that treatment with HA-CS-NAG resulted in a significantly lower MMP-3 concentration than for cells treated with HA alone. In addition, the COX-2 concentration was lower for cells treated with HA-CS-NAG than for the unchallenged negative control cells.

Discussion

The study reported here was focused on elucidating in vitro cellular effects of HA or HA in combination with 2 other compounds (CS and NAG) that have anti-inflammatory or LPS-protective potential on synovial cell death and inflammation. Similar to results of previous investigations, exposure of equine synovial fibroblasts to LPS induced considerable cell injury and cell death as well as release of inflammatory and degradative mediators. The study reported here included evaluation of LPS doses that were similar (20 ng/mL) and greater than (50 ng/mL) previously published doses, and the present study confirmed that negative effects on cells are related to the LPS concentration. The data indicated that cells treated with HA or HA-CS-NAG before and after LPS challenge exposure had significantly better survivability and recovery as well as lower concentrations of inflammatory mediators and degradative enzymes. Analysis of the data suggested that HA may have had slightly greater direct cellular protective effects against high concentrations of LPS, and HA-CS-NAG had greater anti-inflammatory effects because HA-CS-NAG had the greatest suppression of PGE_2, IL-6, and MMP-3 release from cells challenged exposed with LPS. Importantly, both HA and HA-CS-NAG had a profound and significant effect against LPS-induced inflammation at both LPS dosages. Therefore, results of the present study indicated that treatment with this formulation of HA or HA-CS-NAG prior to LPS challenge exposure had supportive effects for synovial cells (including decreased apoptosis and inflammatory mediator production) that would be supportive of an improved joint environment.

One mechanism that may explain the protective findings against LPS is the viscous nature of HA, which is known for sustaining mechanical stress and forces applied to the joints, because it can protect surface-active phospholipids from lysis by phospholipase A2. This suggests an inert physical barrier may be created that limits cellular interaction with LPS, as has been suggested in a previous study[21] that involved use of these same methods. It is plausible that the addition of the other compounds (CS and NAG) may dilute the influence of HA to a minor extent, which would be sufficient to reduce the barrier effect evident as better protection of morphology and higher cell viability. Contrastingly, HA-CS-NAG had the greatest influence on preventing the inflammatory and degradative arm of LPS-induced inflammation, which may have been a result of an additive effect of the multiple anti-inflammatory compounds.

Chondroitin sulfate, GAGs, and HA have all been implicated in inflammatory reactions, with documented roles in synovial membrane inflammation, cell infiltration and activity, biochemical mediator release, and angiogenesis related to osteoarthritis.[13,28–31]

Chondroitin sulfate has catabolic and anabolic activity, which increases synthesis of key components such as HA, glucosamine, and type II collagen while also inhibiting degradative enzymes.[28,29] Similarly, it has been suggested that NAG has an active role in the modification of inflammation and cartilage metabolism, with its key anabolic effects including MMP inhibition as well as functioning as a provider of nutrients to synoviocytes and chondrocytes.[13,28–31] Numerous in vitro studies[31–34] have provided details about the chondroprotective effects of glucosamine and CS, with intra-articular injections of NAG being superior to intra-articular injections of HA for reducing synovitis and cartilage degradation. Maintaining the extracellular matrix and proteoglycan quantity is a critical aspect of the treatment of early osteoarthritis, as is maintaining hydraulic properties of joints through the GAG sequencing components of aggrecan.[31] Therefore, new treatments for osteoarthritis have focused on PG preservation and synthesis.

The objective of the present study was to investigate the effect of treatment with HA on synovial inflammation, particularly evaluation of the protective anti-inflammatory effects of HA and synergistic or combination effects of HA combined with CS and NAG. Although coculture of cartilage and synovium would provide information about the effects of synovial inflammation on articular cartilage degradation, the scope of such a project was beyond the limitations of this study; however, such experiments may reveal other differences in these 2 potential treatments for joint inflammation. In the study reported here, synovial cells were treated with the anti-inflammatory product before challenge exposure with LPS. Other studies[19,22] have focused on improvements attributable to HA after cartilage injury or onset of osteoarthritis. The design of the present study was selected because, to our knowledge, no other studies have been conducted to closely evaluate this anti-inflammatory effect, and we wanted to ensure the product had the best chance to provide an effect, if one existed. In addition, LPS is an extremely potent inflammatory stimulus, and we anticipated that it would be difficult to suppress the effects of LPS. The goal was to enable comparison of results for a previous study[21] conducted by our research group by use of the same methods with results for the present study but with a different HA formulation that has been in clinical use for numerous years. Therefore, conclusions have been limited to a protective effect, but this will still be relevant for many clinical applications. Many sporting horses with noninflammatory chronic joint disease such as osteoarthritis are at risk of acute joint injury and inflammation and could benefit from protective treatments. Also, injections administered after surgery may reduce the onset and intensity of postoperative inflammation.

Limitations of the study reported here with regard to clinical application of intra-articular treatments include the in vitro nature of the study and the fact that LPS induces joint inflammation that differs from the pathophysiology of naturally occurring osteoarthritis. The study design incorporated the use of synovium in which fibroblast-like synoviocytes were the main cell type. Although other cell types are present, culture for 3 or 4 passages results in fibroblast-like synoviocytes from the synovial intimal lining as the dominant cell type of a homogenous population.[35] Cell lines are known to play a key role in cytokine production, inflammation, and secondary cartilage injury; hence, the cells may reflect the in vivo response of a joint. In addition, an in vitro environment is an additional challenge, considering that joints can rapidly replace synovial fluid in vivo, which eliminates inflammatory mediators but also removes therapeutic compounds at the same time. Use of in vitro methods allows control of some of these limitations, compared with a study conducted on joints in vivo. Future in vitro studies by use of synovium and articular cartilage coculture methods could enable further evaluation of the effects of combination treatments on cartilage. Certainly, in vivo studies would provide important information on HA-CS-NAG and HA with regard to intra-articular treatment for inflammation, reductions in degradative enzymes, and potential amelioration of detrimental secondary effects on articular cartilage.

Corticosteroids and NSAIDs, although commonly used for treatment of osteoarthritis, can be limited in their ability to mitigate joint inflammation and degradation.[15] A large number of in vitro and in vivo investigations have been performed, with emphasis placed on the use of nutraceuticals with the potential for disease modification, including products such as pentosan polysulfate, HA, CS, and NAG.[9,18,19] Administration of pentosan polysulfate, which is in clinical use in Australia and New Zealand, alone or in combination with other drugs can inhibit inflammatory mediators such as MMPs and IL-1 as well as promote the synthesis of HA; however, information on the efficacy is limited.[36–38] Anti-inflammatory and disease-modifying effects of HA have been reported, and HA is generally believed to be involved in regulation of PGE_2, upregulation of MMP, and scavenging of oxygen-derived free radicals and to have cartilage-sparing effects.[15] There are a large number of publications on available treatments; however, the lack of randomized, blinded clinical trials and a limited number of systematic reviews limit the direct comparisons that can be made.[39]

For the study reported here, HA and HA-CS-NAG protected synovial cells from the toxic and proinflammatory effects of LPS. In vivo protective effects are suggested on the basis of results for horses with experimentally induced osteoarthritis.[19] An HA-CS-NAG combination may be of value for providing anti-inflammatory effects in horses with synovitis.

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Footnotes

a. Euthasol, Virbac AH, Fort Worth, Tex.
b. Life Technologies, Carlsbad, Calif.
c. Sigma-Aldrich Corp, St Louis, Mo.
d. Antibiotic-antimycotic, Life Technologies, Carlsbad, Calif.
e. Type II collagenase, Life Technologies, Carlsbad, Calif.
f. 70-µm nylon mesh cell strainer, Fisher Scientific, Pittsburgh, Pa.
g. Fisher Scientific, Pittsburgh, Pa.
h. Map-5, Bioniche Animal Health, Pullman, Wash.
i. Polyglycan, ArthroDynamics Inc, Lexington, Ky.
j. *Escherichia coli* O55:B5, Sigma-Aldrich Corp, St Louis, Mo.
k. Gey's balanced salt solution, Sigma-Aldrich Corp, St Louis, Mo.
l. Trypan blue solution (0.4%), Sigma-Aldrich Corp, St Louis, Mo.
m. R&D Systems, Minneapolis, Minn.
n. SpectraMax M2 microplate reader, Molecular Devices, Sunnyvale, Calif.
o. GraphPad Prism, version 6.0 for Macintosh, GraphPad Software Inc, San Diego, Calif.

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