

Effects of serum and autologous conditioned serum on equine articular chondrocytes treated with interleukin-1 β

Eric R. Carlson, DVM; Allison A. Stewart, DVM, MS; Kelly L. Carlson, DVM; Sushmitha S. Durgam, DVM, MS; Holly C. Pondenis, BS

Objective—To compare the effects of autologous equine serum (AES) and autologous conditioned serum (ACS) on equine articular chondrocyte metabolism when stimulated with recombinant human (rh) interleukin (IL)-1 β .

Sample—Articular cartilage and nonconditioned and conditioned serum from 6 young adult horses.

Procedures—Cartilage samples were digested, and chondrocytes were isolated and formed into pellets. Chondrocyte pellets were treated with each of the following: 10% AES, 10% AES and rhIL-1 β , 20% AES and rhIL-1 β , 10% ACS and rhIL-1 β , and 20% ACS and rhIL-1 β , and various effects of these treatments were measured.

Results—Recombinant human IL-1 β treatment led to a decrease in chondrocyte glycosaminoglycan synthesis and collagen II mRNA expression and an increase in medium matrix metalloproteinase-3 activity and cyclooxygenase-2 mRNA expression. When results of ACS and rhIL-1 β treatment were compared with those of AES and rhIL-1 β treatment, no difference was evident in glycosaminoglycan release, total glycosaminoglycan concentration, total DNA content, or matrix metalloproteinase-3 activity. A significant increase was found in chondrocyte glycosaminoglycan synthesis with 20% AES and rhIL-1 β versus 10% ACS and rhIL-1 β . The medium from ACS and rhIL-1 β treatment had a higher concentration of IL-1 β receptor antagonist, compared with medium from AES and rhIL-1 β treatment. Treatment with 20% ACS and rhIL-1 β resulted in a higher medium insulin-like growth factor-I concentration than did treatment with 10% AES and rhIL-1 β . No difference in mRNA expression was found between ACS and rhIL-1 β treatment and AES and rhIL-1 β treatment.

Conclusions and Clinical Relevance—Minimal beneficial effects of ACS treatment on proteoglycan matrix metabolism in equine chondrocytes were evident, compared with the effects of AES treatment. (*Am J Vet Res* 2013;74:700–705)

Osteoarthritis, which is a common cause of lameness and poor performance in horses,^{1–5} can be influenced by a horse's conformation, age, use, exercise regimen, and other factors. The goal of osteoarthritis treatment in performance horses is to alleviate signs of pain while, when appropriate, allowing those horses to continue training or competition. Several mechanical and biological dysfunctions can develop in affected joints. Osteoarthritis-associated cartilage degradation is mediated in part by cytokines and growth factors. Interleukin-1, which is a catabolic cytokine, is a potent known mediator of cartilage loss that can lead to osteoarthritis.⁶ The cytokine induces a cascade of inflammatory and catabolic events, including the expression of

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From the Department of Veterinary Clinical Medicine, College of Veterinary Medicine, University of Illinois, Urbana, IL 61802.

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Address correspondence to Dr. Stewart (aaw@illinois.edu).

ABBREVIATIONS

ACS	Autologous conditioned serum
AES	Autologous equine serum
COX	Cyclooxygenase
GAG	Glycosaminoglycan
IGF	Insulin-like growth factor
IL	Interleukin
IL-1RA	Interleukin-1 β receptor antagonist
MMP	Matrix metalloproteinase
rh	Recombinant human

cartilage-degrading MMPs, production of nitric oxide, release of prostaglandin E₂, and inhibition of proteoglycan and collagen synthesis.^{7–15}

The naturally occurring inhibitor of IL-1, IL-1RA, could potentially limit the actions of IL-1 and thereby minimize the progression of osteoarthritis. Several investigators have reported a decrease in lameness with intra-articular injection of IL-1RA in several experimental models of osteoarthritis and rheumatoid arthritis in animals.^{12,15} Another means of producing IL-1RA for intra-articular administration is ACS, which can be commercially prepared from equine blood.^{11,16}

Autologous conditioned serum is prepared from whole blood incubated with medical grade borosilicate glass beads to initiate monocyte activation.¹¹ In humans, serum prepared with the ACS method contains an increased concentration of IL-1RA, IL-4, and IL-10 over time.⁷ In addition, intra-articular administration of ACS can achieve a limited to moderate decrease in lameness severity in humans with osteoarthritis.¹⁰ In horses with experimentally induced osteochondral fragments, the ACS-treated group had significant improvement in signs of lameness on day 70, compared with the placebo-treated group.¹¹

Two ACS products are marketed for use in horses. In 1 study,¹⁶ equine serum prepared with one ACS system yielded a significant moderate increase in serum IL-1RA concentration, compared with serum prepared with another ACS product or nonconditioned serum. That study¹⁶ also showed higher concentrations of IGF-1 and tumor necrosis factor- α protein were present in both ACS samples. Specifically, greater concentrations of IL-10 and transforming growth factor- β than in nonconditioned serum were detected in serum obtained with the ACS systems and also in nonconditioned serum incubated in borosilicate glass tubes containing a clot activator, indicating that upregulation may be a consequence of whole blood culture rather than treatment with the ACS systems.¹⁶ That study¹⁶ also showed that addition of heparin to serum samples led to a significant decrease in concentrations of IGF-1, tumor necrosis factor- α , and transforming growth factor- β and, conversely, an increase in IL-1RA concentration. These findings suggest that changes in the cytokine concentrations may be dependent on the clotting cascade, indicating the involvement of platelet degranulation.¹⁶

The purpose of the study reported here was to evaluate the effects of AES and ACS on GAG synthesis and metabolism *in vitro* by use of pellets of cultured chondrocytes. The null hypothesis was that there would be no difference in chondrocyte pellet matrix metabolism and inflammatory mediators when chondrocyte treatments with ACS and AES were compared.

Materials and Methods

Horses and sample collection—Samples used in the study were obtained from 6 systemically healthy horses aged 1 to 4 years that were to be euthanized for reasons unrelated to joint disease. Before euthanasia, each horse had a jugular vein aseptically prepared and 60 mL of blood was aspirated into a borosilicate glass tube containing a clot activator. The samples were allowed to clot and then were centrifuged, and the serum was transferred via pipette to a plastic test tube. A blood sample was also collected in an ACS syringe and processed in accordance with the syringe manufacturer's instructions.^a Both types of samples were stored at -80°C .

After horses were euthanized via a lethal dose of sodium pentobarbital administered IV, all joints were evaluated to ensure there was no gross evidence of joint disease. Afterward, samples of articular cartilage were aseptically collected from the stifle joint. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Illinois.

Chondrocyte culture—On the day of sample collection (day 0), samples of articular cartilage were placed in chondrogenic medium consisting of Dulbecco modified Eagle medium,^b 10% AES, 300 μg of L-glutamine/mL,^c 100 U of sodium penicillin/mL, 100 μg of streptomycin sulfate/mL,^d and ascorbic acid^e (50 μg /mL) and were digested overnight with 0.2% collagenase.^f After the digestion process, total chondrocyte number and viability were estimated with a Reichart hemacytometer and the trypan blue exclusion test.^{17,g}

Isolated chondrocytes were suspended at a concentration of 500,000 cells/mL in chondrogenic medium, transferred to an Eppendorf tube (0.5 mL/tube), and centrifuged to form chondrocyte pellets containing 250,000 cells. The pellets were incubated at 37°C for 6 days to allow formation of an extracellular matrix, and the medium was changed every 2 to 3 days.

Chondrocyte pellet treatments—Twenty-three chondrocyte pellets were used for each treatment group. Chondrocyte pellets underwent 5 treatments on days 2 and 5 after cartilage sample collection: 10% AES (negative control treatment), rhIL-1 β^{h} (10 ng/mL) and 10% AES (positive control), rhIL-1 β and 20% AES, rhIL-1 β and 10% ACS,^a and rhIL-1 β and 20% ACS. Three pellets from each treatment group were radiolabeled with sulfate 35 ($^{35}\text{SO}_4$) on day 5 and incubated for 24 hours to measure GAG synthesis.¹⁸ Pellets and media were collected on day 6.

Measurement of pellet GAG synthesis—Glycosaminoglycan synthesis was determined via $^{35}\text{SO}_4$ incorporation into the pellet and subsequent release into the medium during a 24-hour period. Three radiolabeled pellets were digested individually in papainⁱ (100 μL /pellet) at 65°C for 24 hours and then precipitated with 0.2% alcian blue dye solution and counted for scintillation.¹⁸ Radioisotope decay was accounted for through the amount of radioisotope in each pellet and the amount released into the medium, and scintillation counts were normalized for total pellet and media digestion volume to determine the amount of GAG release into the medium.

Measurement of pellet total GAG content—Total GAG content in 3 pellets and related medium from each treatment group was determined through use of a 1,9-dimethylmethylene blue binding assay.^{19,j} All sample values were compared against a standard curve of chondroitin sulfate values to estimate the total GAG content and normalized for pellet digestion volume.

Measurement of pellet total DNA content—Total DNA content of each pellet was determined by use of a fluorometric Hoechst assay.^{20,k} Three pellets were individually digested in papain, dye solution was added, and samples were analyzed for fluorescence in a microplate reader.^l All sample values were compared against a standard curve of calf thymus values to estimate total DNA content of the digested pellets.

Measurement of medium MMP-3 activity—Matrix metalloproteinase-3 activity in pellet medium was determined with an ELISA kit^m in accordance with the manufacturer's protocol. Briefly, a standard sample, control sample, or medium sample was com-

bined with an MMP-3 conjugate. The samples were washed and incubated with a substrate solution, and optical density was measured with a microplate reader set to 450 nm.

Measurement of medium IGF-I concentration—Insulin-like growth factor-I concentration in pellet medium was determined with an ELISA kitⁿ validated for use with equine samples. Again, a standard sample, control sample, or medium sample was incubated with an IGF-I conjugate. The samples were washed and combined with a substrate solution, and optical density was measured with a microplate reader set to 450 nm.

Measurement of medium IL-1RA concentration—Interleukin-1 β receptor antagonist concentration in pellet medium was measured with an equine-specific ELISA kit.^o Briefly, the capture antibody was incubated with each sample. The samples were washed and combined with the detection antibody prior to incubation with streptavidin-horseradish peroxidase. Substrate solution was added to each, and the optical density was measured with a microplate reader set to 450 nm.

Measurement of pellet mRNA content—The RNA was extracted from 15 pellets from each of 5 horses/treatment by use of a reagent^p and RNA isolation kit^q in accordance with the manufacturer's suggested protocol. Complementary DNA was obtained by priming the sample with oligo d(T)^r and then adding reverse transcriptase. Real-time quantitative PCR analysis^s was performed for MMP-3, collagen type II, aggrecan, and COX-2, and results were normalized to elongation factor-1 α mRNA expression.

Statistical analysis—A sample size of 6 was necessary to detect differences in media. The concentration of all analytes in the medium of chondrocyte pellets treated with AES and rhIL-1 β was compared with that of pellets treated with ACS and rhIL-1 β . All nonnormally distributed data were logarithmically transformed and are presented as mean \pm SD log values. One-way repeated-measures ANOVA was performed with a software program^t to compare the positive control (10% AES and rhIL-1 β) values with the negative control (10% AES) values. Values for the AES and rhIL-1 β treatment and the ACS and rhIL-1 β treatment were similarly compared. Post hoc tests were conducted when indicated by means of the Holm-Sidak method. Values of $P \leq 0.05$ were considered significant for all analyses.

Results

GAG synthesis and release—Treatment of equine chondrocyte pellets with rhIL-1 β resulted in a significant ($P = 0.024$) decrease in pellet GAG synthesis, as inferred by comparison of values for the positive control (10% AES and rhIL-1 β) treatment with those of the negative control (10% AES) treatment (Table 1). A significant ($P = 0.008$) increase in pellet GAG synthesis occurred with the 20% AES and rhIL-1 β treatment, compared with synthesis with the 10% ACS and rhIL-1 β treatment. No significant difference in pellet GAG synthesis was identified among the remaining treatment groups. When the amount of GAG release into pellet medium was measured, there was no significant difference between the negative and positive control treatments, but there was a nonsignificant ($P = 0.054$) increase in GAG release with rhIL-1 β treatment. No significant differences were evident among the remaining treatments for GAG release.

Pellet total GAG concentration—That effect of rhIL-1 β treatment was a nonsignificant ($P = 0.054$) decrease in total pellet GAG concentration in the negative versus positive control groups (Table 1). No significant difference in total pellet GAG concentration was found among the remaining treatment groups. No significant difference in medium total GAG concentration was evident between negative and positive control treatments. There were also no significant differences among the remaining treatments with respect to total GAG concentration in the pellet medium.

Pellet DNA—No significant difference in pellet DNA content was found between negative and positive control treatments or among the remaining treatments (Table 1).

MMP-3—Treatment of chondrocyte pellets with rhIL-1 β resulted in a significant ($P = 0.002$) increase in medium MMP-3 concentration as revealed through comparison of results for the positive and negative control treatments. The concentration did not differ between AES and rhIL-1 β treatment and ACS and rhIL-1 β treatment (Table 2).

IGF-I—The positive and negative control treatments did not differ with respect to medium IGF-I concentration. However, the IGF-I concentration was significantly higher in medium from pellets treated with 20% ACS and rhIL-1 β than with the positive control

Table 1—Log mean \pm SD values for the effects of various treatments on radiolabeled sodium sulfate incorporation into GAG in chondrocyte pellets and medium, total GAG production in chondrocyte pellets and medium, and total amount of DNA in pellets of chondrocytes obtained from articular cartilage of stifle joints in 6 young adult horses.

Treatment	GAG incorporation (cpm/pellet)	GAG incorporation (cpm/mL of medium)	Total GAG in pellet (μ L/mL)	Total GAG in medium (μ L/mL)	Total DNA (μ L/pellet)
10% AES (negative control)	3.509 \pm 0.20 ^a	3.175 \pm 0.38	1.524 \pm 0.23	1.591 \pm 0.15	0.442 \pm 0.30
10% AES and rhIL-1 β (positive control)	3.212 \pm 0.17 ^{b,c}	2.988 \pm 0.32	1.309 \pm 0.20	1.564 \pm 0.19	0.422 \pm 0.27
20% AES and rhIL-1 β	3.266 \pm 0.24 ^b	2.906 \pm 0.27	1.415 \pm 0.22	1.554 \pm 0.26	0.519 \pm 0.25
10% ACS and rhIL-1 β	3.126 \pm 0.24 ^c	2.914 \pm 0.29	1.356 \pm 0.18	1.469 \pm 0.16	0.448 \pm 0.33
20% ACS and rhIL-1 β	3.143 \pm 0.19 ^{b,c}	2.884 \pm 0.26	1.368 \pm 0.22	1.596 \pm 0.29	0.520 \pm 0.27

Original values before logarithmic transformation were measured in counts per million (cpm), μ L/pellet, and μ L/mL.
^{a-c}Within a column, values with different superscript letters are significantly ($P < 0.05$) different.

Table 2—Log mean \pm SD values for the effects of various treatments on MMP-3 activity and IGF-1 and IL-1RA concentrations in culture medium for pellets of chondrocytes obtained from articular cartilage of stifle joints in 6 young adult horses.

Treatment	MMP-3	IGF-1	IL-1RA
10% AES (negative control)	0.40 \pm 0.07 ^a	-0.22 \pm 0.16 ^a	3.40 \pm 0.36 ^a
10% AES and rhIL-1 β (positive control)	0.51 \pm 0.11 ^b	-0.10 \pm 0.28 ^a	3.83 \pm 0.34 ^a
20% AES and rhIL-1 β	0.53 \pm 0.10 ^b	0.08 \pm 0.31 ^{a,b}	3.94 \pm 0.26 ^a
10% ACS and rhIL-1 β	0.48 \pm 0.09 ^b	-0.01 \pm 0.37 ^a	4.34 \pm 0.08 ^b
20% ACS and rhIL-1 β	0.53 \pm 0.09 ^b	0.18 \pm 0.41 ^b	4.38 \pm 0.08 ^b

Original values before logarithmic transformation were measured in pg/mL.
See Table 1 for remainder of key.

Table 3—Mean \pm SD degree of mRNA expression for the effects of various treatments on MMP-3, COX-2, collagen type II, and aggrecan in chondrocyte pellets obtained from articular cartilage in stifle joints of 5 young adult horses.

Treatments	Coll-II	Aggrecan	COX-2	MMP-3
10% AES (negative control)	1 \pm 0.0	1 \pm 0.0	1 \pm 0.0	1 \pm 0.0
10% AES and rhIL-1 β (positive control)	0.21 \pm 0.08*	1.06 \pm 0.82	94.70 \pm 75.94*	223.10 \pm 241.61
20% AES and rhIL-1 β	0.14 \pm 0.09	0.55 \pm 0.58	86.69 \pm 86.61	202.16 \pm 286.06
10% ACS and rhIL-1 β	0.19 \pm 0.14	0.91 \pm 0.65	246.73 \pm 384.25	250.72 \pm 340.37
20% ACS and rhIL-1 β	0.28 \pm 0.34	0.42 \pm 0.10	21.31 \pm 18.54	162.08 \pm 117.98

*Value differs significantly ($P < 0.05$) from that for 10% AES alone.

(10% AES and rhIL-1 β ; $P = 0.001$) and 10% ACS and rhIL-1 β ($P = 0.01$) treatments (Table 2).

IL-1RA—Medium IL-1RA concentrations were similar between positive and negative control treatments. Media from chondrocyte pellets treated with ACS and rhIL-1 β had a significantly ($P < 0.001$) higher IL-1RA concentration than did those treated with AES and rhIL-1 β (Table 2).

Pellet mRNA content—Treatment with rhIL-1 β resulted in a significant ($P < 0.001$) decrease in collagen type II mRNA expression and significant ($P < 0.001$) increase in COX-2 mRNA expression, as shown by comparison of results for the positive versus negative control treatment. No significant effect on either type of mRNA expression was found when comparing values for the AES and rhIL-1 β and ACS and rhIL-1 β treatments. Aggrecan and MMP-3 mRNA expression did not differ between negative and positive control treatments or among the other treatments (Table 3).

Discussion

The composition of GAG within the cartilage matrix is dependent on a balance of synthetic and degradative processes.²¹ Glycosaminoglycan binds a large volume of water, giving articular cartilage its unique compressive biomechanical behavior. The present study showed that treatment of chondrocytes from the articular cartilage of horses with IL-1 β resulted in a decrease in the amount of newly synthesized GAG in the pellet, compared with the amount in chondrocytes treated with 10% AES. These findings are in agreement with those of a previous study¹⁷; however, both studies revealed no difference in the amount of synthesized GAG released into the chondrocyte medium with IL-1 β treatment.¹⁷

A significant increase in pellet GAG synthesis occurred when equine chondrocyte pellets were treated

with 20% AES and rhIL-1 β , compared with when they were treated with 10% ACS and rhIL-1 β , but there was no significant difference in this variable among the other treatment groups. These findings may not be scientifically relevant because other study variables did not further support the findings. Specifically, pellet treatment with AES resulted in similar concentrations of inflammatory mediators, total pellet GAG content, and aggrecan mRNA, compared with treatment with ACS. In several studies,^{22,23} supplementation of cell culture medium with 20% serum yielded better results than with $< 10\%$ serum for cell proliferation and matrix development. In our study, the impact of increasing the concentrations of AES or ACS from 10% to 20% without IL-1 β supplementation on GAG metabolism could not be assessed because controls containing 20% serum without IL-1 β were not included. Regardless, when GAG synthesis and release into culture medium were evaluated in the presence of IL-1 β , treatment with ACS was not superior to treatment with AES. These findings support our null hypothesis that treatment with ACS would not improve chondrocyte proteoglycan metabolism, compared with treatment with a similar amount of AES.

Pellet treatment with IL-1 β had several detrimental effects on chondrocyte metabolism in the present study. Addition of IL-1 β resulted in a decrease in newly synthesized GAG in the pellet, compared with the amount in pellets with the negative control treatment, and this observation was similar to that in a previous study.¹⁷ Treatment with IL-1 β also led to an increase in COX-2 mRNA expression relative to the negative control treatment, as was found in the same previous study.¹⁷ In addition, the IL-1 β treatment caused a decrease in collagen type II mRNA expression and increase in medium MMP-3 concentration relative to negative control values. Matrix metalloproteinase-3 can be localized to cartilage zones with active proteoglycan depletion. Interleukin-1 β induces synthesis of collagenase and

stromelysin (MMP-3) and a decrease in secretion of tissue inhibitor of metalloproteinases.¹⁰ This could explain the decrease in the amount of synthesized GAG retained in chondrocyte pellets in the present study.

Treatment with IL-1 β had no apparent effect on total pellet and medium GAG concentrations in our study. Another study²¹ also failed to demonstrate an effect of IL-1 on total GAG release into culture medium, potentially because of the complex effects of these cytokines on cartilage metabolism. When the ACS and AES treatments were compared, there was no statistical difference in total pellet and medium GAG concentrations when stimulated with IL-1 β . An *in vivo* study¹¹ in horses yielded similar results; no difference was found in total GAG concentration between ACS- and saline (0.9% NaCl) solution-treated osteoarthritic joints. An *in vitro* human study²⁴ also showed no difference in total GAG concentration between cartilage explants from people with osteoarthritis of the knee joint treated with serum or ACS. The present *in vitro* study involving equine chondrocytes obtained from stifle joints revealed no significant improvement in total matrix GAG concentrations when the cells were treated with ACS versus AES alone.

Proteins of the MMP family are involved in the breakdown of extracellular matrix during remodeling in healthy and arthritic tissues. Matrix metalloproteinase-3 degrades proteoglycans, fibronectin, laminin, and elastin. In addition, the enzyme can activate other MMPs such as MMP-1, MMP-7, and MMP-9. Plasma activities of MMP-3 are reportedly significantly higher in humans with osteoarthritis than in healthy subjects.²⁵ In the present study, there was no significant difference between ACS and AES treatments; however, there was a significant increase in medium MMP-3 activity when the pellet was treated with IL-1 β . These findings are similar to those of another study²⁶ that showed chondrocyte medium supplementation with IL-1 β at 10 ng/mL increased medium activities of MMP-3 and MMP-13.

Insulin-like growth factor-I plays a key role in cartilage homeostasis, balancing proteoglycan synthesis and breakdown.^{27,28} The suggestion that IGF-I is required to maintain articular cartilage integrity is supported by findings that rats with chronic IGF-I deficiency develop articular cartilage lesions.²⁷ The growth factor can repair extensive cartilage defects in experimental disease models.²⁸ In the present study, treatment of equine chondrocyte pellets with 20% ACS and rhIL-1 β yielded a significant increase in IGF-I concentration relative to that achieved with 10% ACS and rhIL-1 β or 10% AES and rhIL-1 β , but no significant difference was identified between 20% ACS and 20% AES treatments. This differed from findings in the study¹⁶ in which cytokine concentrations in 2 ACS products and serum final products were compared. In that study,¹⁶ ACS from both ACS products resulted in significant increases in IGF-I, compared with results for nonconditioned serum at 1 and 24 hours after serum separation. The differences between those findings and ours may be explained by differences in study design. In the present study, 10% or 20% AES or ACS was used and differences in medium concentrations were evaluated 24 hours following incubation with articular chondrocytes and rhIL-1 β . In

the other study,¹⁶ the mediators contained within 100% AES or ACS were evaluated without any exposure to cells or IL-1. In essence, our study started with a smaller percentage of serum in culture medium and had the additive effect of exposure to chondrocytes and inflammation, which could lead to a reduction in medium IGF-I concentration.

The present study supported the increased production of IL-1RA when the effects of one of the ACS products^a used in the other study¹⁶ were compared with the effects of nonconditioned serum. However, the increases did not lead to an anabolic shift in proteoglycan metabolism. Our findings suggest that the effect of nonconditioned serum is equivalent to the ACS product^a when IL-1 β is used. It would have been interesting to have obtained serum through use of plastic tubes and compare the findings with use of the borosilicated glass tubes containing a clot activator and immediate centrifugation or incubation for 24 hours.

No significant differences were found in mRNA expression between AES and ACS treatments for COX-2, collagen type II, MMP-3, and aggrecan. Chondrocyte treatment with IL-1 β significantly increased mRNA expression of COX-2 and decreased expression of collagen type II but had no effect on mRNA expression of MMP-3 and aggrecan. In another study,²¹ equine chondrocytes were treated with various concentrations of IL-1, showing that IL-1 exposure led to a decrease in the amount of collagen type II mRNA expression. That study²¹ also identified an increase in mRNA expression of MMP-3 and decrease in aggrecan treatment with IL-1 treatment. In the present study, an increase in MMP-3 mRNA expression did occur with medium rhIL-1 β supplementation, but this increase was not significant because of a large SD.

The present study had several limitations. Chondrocytes from only 6 horses were used, which was the minimum number estimated as necessary to detect differences in medium IL-1RA concentrations with ACS treatment. A larger number of horses (14 to 23, as calculated) may have allowed detection of differences between ACS and AES in proteoglycan metabolism if such differences do exist. Use of ACS may also mediate or modify the development of osteoarthritis through pathways not evaluated in our study. The ACS and AES used might have had an abundance of growth factors that made it difficult to detect a difference in chondrocyte proteoglycan metabolism. A coculture of synoviocytes and chondrocytes could be used to more accurately represent cells involved in osteoarthritis and demonstrate differences in chondrocyte and synoviocyte metabolism not detected in our study.

Other limitations include the fact that the chondrocyte pellets used were only treated with IL-1 β , which is reportedly effective in a model of inflammation using articular chondrocytes but does not fully replicate naturally occurring osteoarthritis. Use of other proinflammatory mediators (tumor necrosis factor- α , IL-6, IL-8, IL-11, IL-17, or leukemia inhibitory factor) in addition to IL-1 β administration might better simulate the conditions in naturally occurring osteoarthritis and might have had an effect on the study findings.

Studies^{8–11} in humans and other animals have shown that ACS administration aids in amelioration of the clinical signs of osteoarthritis, but its effects have not been compared directly with those of AES administration. More research is needed to determine the characteristics of AES that yield results similar to ACS. Because osteoarthritis and equine joints are complex, the findings reported here may differ, should other tissues such as synovium be used. Although chondrocyte treatment with ACS did lead to an increase in culture medium IL-1RA concentration in the present study, it did not lead to a direct effect on in vitro proteoglycan cartilage metabolism. Furthermore, ACS had no significant effect on MMP-3 mRNA expression, compared with AES. It may be that ACS exerts its effects through mechanisms other than those that were evaluated in the present study. Many aspects of ACS treatment remain to be elucidated.

- a. IRAP II, Arthrex, Naples, Fla.
- b. Mediatech Inc, Herndon, Va.
- c. L-glutamine, 200mM, Invitrogen, Carlsbad, Calif.
- d. Penicillin-streptomycin, BioWhittaker, Cambrex Bio Science, Walkerville, Md.
- e. Ascorbic acid, WAKO, Richmond, Va.
- f. Collagenase type 2, Worthington Biomedical Corp, Lakewood, NJ.
- g. Invitrogen, Carlsbad, Calif.
- h. IL-1 β , R&D Systems, Minneapolis, Minn.
- i. Papain, Sigma-Aldrich, St Louis, Mo.
- j. 1,9-dimethyl-methylene blue, Sigma-Aldrich, St Louis, Mo.
- k. Hoechst 33258, Sigma-Aldrich, St Louis, Mo.
- l. Microplate reader, FLUOstar Optima, BMG Laboratories, Durham, NC.
- m. MMP-3, R&D Systems, Minneapolis, Minn.
- n. IGF-1, R&D Systems, Minneapolis, Minn.
- o. IL-1 β Ra, R&D Systems, Minneapolis, Minn.
- p. Trizol, Invitrogen, Carlsbad, Calif.
- q. RNeasy, Qiagen, Valencia, Calif.
- r. Oligo d(T), Invitrogen, Carlsbad, Calif.
- s. iCycler iQ real-time PCR detection system, Bio-Rad Laboratories, Hercules, Calif.
- t. SigmaStat, version 3.0, Systat Software Inc, San Jose, Calif.

References

1. USDA APHIS Veterinary Services National Health Monitoring System. *National economic cost of equine lameness, colic, and equine protozoal myeloencephalitis in the United States*. Information Sheet. No. N348.1001. Fort Collins, Colo: USDA APHIS Veterinary Services National Health Monitoring System, 2001.
2. Jeffcott LB, Rossdale PD, Freestone J, et al. An assessment of wastage in Thoroughbred racing from conception to 4 years of age. *Equine Vet J* 1982;14:185–198.
3. Rossdale PD, Hopes R, Digby NJ, et al. Epidemiological study of wastage among racehorses 1982 and 1983. *Vet Rec* 1985;116:66–69.
4. Neundorff RH, Lowerison MB, Cruz AM, et al. Determination of the prevalence and severity of metacarpophalangeal joint osteoarthritis in Thoroughbred racehorses via quantitative macroscopic evaluation. *Am J Vet Res* 2010;71:1284–1293.
5. Jackman BR. Common lameness in the cutting and reining horse, in *Proceedings*. 51st Annu Conv Am Assoc Equine Pract 2001;6–11.
6. Joek N. Lameness in the western pleasure horses, in *Proceedings*. 51st Annu Conv Am Assoc Equine Pract 2001;12–14.
7. Smith RL, Allison AC, Schurman DJ. Induction of articular cartilage degradation by recombinant interleukin 1 alpha and 1 beta. *Connect Tissue Res* 1989;18:307–316.
8. Meijer H, Reinecke J, Becker C, et al. The production of anti-inflammatory cytokines in whole blood by physico-chemical induction. *Inflammation Res* 2003;52:404–407.
9. Malyak M, Swaney RE, Arend WP. Levels of synovial fluid interleukin-1 receptor antagonist in rheumatoid arthritis and other arthropathies. Potential contribution from synovial fluid neutrophils. *Arthritis Rheum* 1993;36:781–789.
10. Martel-Pelletier J, Pelletier JP. Importance of interleukin-1 receptors in osteoarthritis [in French]. *Rev Rhum Ed Fr* 1994;61:109–113.
11. Frisbie DD, Kawcak CE, Werpy NM, et al. Clinical, biochemical, and histologic effects of intra-articular administration of autologous conditioned serum in horses with experimentally induced osteoarthritis. *Am J Vet Res* 2007;68:290–296.
12. Caron JP, Fernandes JC, Martel-Pelletier J, et al. Chondroprotective effect of intraarticular injections of interleukin-1 receptor antagonist in experimental osteoarthritis. Suppression of collagenase-1 expression. *Arthritis Rheum* 1996;39:1535–1544.
13. Stove J, Huch K, Gunther KP, et al. Interleukin-1beta induces different gene expression of stromelysin, aggrecan and tumor-necrosis-factor-stimulated gene 6 in human osteoarthritic chondrocytes in vitro. *Pathobiology* 2000;68:144–149.
14. Goldring MB, Berenbaum F. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. *Clin Orthop Relat Res* 2004;427(suppl):S37–S46.
15. Frisbie DD, Ghivizzani SC, Robbins PD, et al. Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther* 2002;9:12–20.
16. Hraha TH, Doremus KM, McIlwraith CW, et al. Autologous conditioned serum: the comparative cytokine profiles of two commercial methods (IRAP and IRAP II) using equine blood. *Equine Vet J* 2011;43:516–521.
17. Schaefer EC, Stewart AA, Durgam SS, et al. Effects of sodium hyaluronate and triamcinolone acetonide on glucosaminoglycan metabolism in equine articular chondrocytes treated with interleukin-1. *Am J Vet Res* 2009;70:1494–1501.
18. Masuda K, Shirota H, Thonar EJ. Quantification of 35S-labeled proteoglycans complexed to alcian blue by rapid filtration in multiwell plates. *Anal Biochem* 1994;217:167–175.
19. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. *Connect Tissue Res* 1982;9:247–248.
20. Kim YJ, Sah RL, Doong JY, et al. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal Biochem* 1988;174:168–176.
21. Grodzinsky AJ, Levenston ME, Jin M, et al. Cartilage tissue remodeling in response to mechanical forces. *Annual Rev Biomed Eng* 2000;2:691–713.
22. Richardson DW, Dodge GR. Dose-dependent effects of corticosteroids on the expression of matrix-related genes in normal and cytokine-treated articular chondrocytes. *Inflamm Res* 2003;52:39–49.
23. Koller MR, Maher RJ, Manchel I, et al. Alternatives to animal sera for human bone marrow cell expansion: human serum and serum-free media. *J Hematother* 1998;7:413–423.
24. Rutgers M, Saris DB, Dhert WJ, et al. Cytokine profile of autologous conditioned serum for treatment of osteoarthritis, in vitro effects on cartilage metabolism and intra-articular levels after injection. *Arthritis Res Ther* 2010;12:R114.
25. Naito K, Takahashi M, Kushida K, et al. Measurement of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in patients with knee osteoarthritis: comparison with generalized osteoarthritis. *Rheumatol (Oxford)* 1999;38:510–515.
26. Wang X, Li F, Fan C, et al. Effects and relationship of ERK1 and ERK2 in interleukin-1B-induced alterations in MMP3, MMP13, type II collagen and aggrecan expression in human chondrocytes. *Int J Mol Med* 2011;27:583–589.
27. Ekenstedt KJ, Sonntag WE, Loeser RF, et al. Effects of chronic growth hormone and insulin-like growth factor 1 deficiency on osteoarthritis severity in rat knee joints. *Arthritis Rheum* 2006;54:3850–3858.
28. Fortier LA, Barker JU, Strauss EJ, et al. The role of growth factors in cartilage repair. *Clin Orthop Relat Res* 2011;469:2706–2715.