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Biochemical effects of two different hyaluronic acid products in a co-culture model of osteoarthritis

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Summary

Objectives: To compare the effects of two hyaluronic acid (HA) formulations on mediators of matrix turnover and inflammation in an IL-1-treated cartilage–synovium co-culture model with the aim of elucidating mechanisms by which viscosupplementation exerts beneficial effects in osteoarthritic joints.

Design: A co-culture model (100 ng/ml interleukin-1β (IL-1β) added to canine synovial and cartilage explants) was used to investigate the effects of HA on cartilage–synovium interactions. Three concentrations (1×, 0.5×, and 0.1×) of two commercial sources of HA (A: Synvisc [hylan G-F 20]; B: Hyalgan [sodium hyaluronate]) were used. Co-cultures without IL-1β (negative) or with IL-1β (positive) but neither HA product served as controls. The liquid media were collected every 3 days and explants of cartilage and synovium were collected on days 3, 6, and 20. Media and explants were analyzed histologically, biochemically, and immunohistochemically.

Results: Glycosaminoglycan (GAG) content was measured in cartilage explants. GAG content in explants was higher in both HA groups at the beginning and the conclusion of the study compared to the IL-1β-treated group. GAG content of the media was significantly ($P < 0.05$) lower in the Synvisc group than all other groups early. The Hyalgan group demonstrated progressively less GAG release later in the study. The addition of Synvisc did not decrease the matrix metalloproteinase (MMP)-3 concentrations at any point. MMP-3 concentrations were significantly ($P < 0.05$) lower among the 1× and 0.5× Hyalgan groups on day 20 compared to the IL-1β-treated group. On day 3, prostaglandin E_2 concentrations were significantly ($P < 0.05$) higher in the IL-1β-treated group compared to other groups. Both HA groups had less nitric oxide production than the control groups throughout the study.

Conclusions: This study supports two potential mechanisms for viscosupplementation: a biosynthetic-chondroprotective mechanism, with a possible delay in onset depending on the form of HA; and an anti-inflammatory mechanism.

Key words: Hyaluronic acid, Interleukin-1β, Osteoarthritis, Viscosupplementation, Chondrocytes.

Background

Osteoarthritis (OA) is among the most frequent and symptomatic medical problems for middle-aged and older people, afflicting an estimated 12% of people aged 25–74 in the United States[1,2]. Numerous risk factors for OA have been identified[3]; however, the exact etiology, pathogenesis, and progression of this disease have yet to be determined.

There has been a shift over the past decade in the conceptualization of OA. It is no longer strictly viewed as a "wear and tear" biomechanical process, but also seen in terms of biochemical and molecular events causing inflammation and degradation[4]. This multifactorial concept stems from the knowledge that cartilage is a dynamic structure and OA represents a sustained imbalance between cartilage synthesis and degradation, favoring the latter.

Articular cartilage functions to provide load transmission across a joint via a relatively frictionless surface. Cartilage is composed of cells (chondrocytes) surrounded by an extracellular matrix (ECM). The ECM consists of water, collagen (mainly type 2), proteoglycans (PGs), and an assortment of other proteins. The PG aggrecan molecule is attached via a link protein to hyaluronic acid (HA). This entire complex is referred to as a PG aggregate. Collagen forms a matrix of fibers that is interwoven with the network of HA. In this way, HA acts as a major aggregating factor between the collagen, PG aggregate, and cartilage structural network as a whole[5].

Synovial fluid contains high concentrations of HA, and HA in synovial fluid is produced by Type B synoviocytes. HA plays a key role in determining the viscoelastic properties of the synovial fluid[6]. Under low-shear stresses, HA provides high viscosity and low elasticity (joint lubricant); when shear stresses become high, HA becomes more elastic and much more efficient at absorbing energy[7].

It has been shown that in advanced stages of OA, there is a more rapid turnover of the ECM and a reduction in all its major components, including HA[8]. Although this turnover is part of the cartilage and synovial tissues' repair response, the process within the tissue is ineffective and the degradative process associated with OA inevitably progresses[3]. Further, the turnover is likely responsible for the decrease in concentration and average molecular weight of the HA observed in the synovial fluid of OA joints, which results in a decrease in the viscoelastic properties of the fluid.

If it were possible to maintain the levels of the components in the ECM, degradation of osteoarthritic cartilage might be ameliorated. While there are a number of clinically proven

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modalities available to alleviate chronic pain and disability associated with OA, no medical intervention has so far been shown to halt disease progression or reverse joint damage in humans[9]. Intra-articular injection of HA, termed viscosupplementation, is a relatively new pharmacologic therapy that has demonstrated statistically and clinically significant improvement of symptoms in patients with OA[10].

HA injected into the joint has a half-life of approximately 20 h when joints are normal and about 12 h when joints are inflamed[11–15]; however, the benefit of intra-articular HA therapy has been shown to last for months.

The *purpose* of this study was to compare the effects of two HA formulations on mediators of matrix turnover and inflammation in an IL-1-treated cartilage–synovium co-culture model with the aim of elucidating mechanisms by which viscosupplementation exerts beneficial effects in osteoarthritic joints.

We hypothesized that HA would have beneficial effects in a canine cartilage–synovium explant model with respect to matrix synthesis and mediators of inflammation and degradation. To test our hypothesis, we used a co-culture model, which has been reported to represent spontaneously occurring OA with respect to gene expression, matrix synthesis, and mediators of inflammation and degradation[16–18].

Materials and methods

SAMPLE COLLECTION

Full-thickness articular cartilage and synovium were obtained from canine cadavers ($n = 4$) from a local humane society after euthanasia performed for reasons unrelated to this study. Articular cartilage was harvested from the proximal humeral head; synovium was harvested from the stifle joint. All joints used were judged to be normal based on gross inspection of articular cartilage, joint capsule, ligaments, and menisci.

CO-CULTURE

Cartilage (4 mm) and synovial discs (6 mm) were prepared using dermal biopsy punches (Fray products corp., Buffalo, NY). Synovium explants were placed in 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) with one explant per well. Filter inserts (0.4 μm pore, Becton Dickinson Labware) were placed in the 24-well plates, and cartilage explants from the same dog were placed in the filter inserts with one explant per insert (Fig. 1). Explants were cultured in 1 mm of RPMI 1640 medium with 10% fetal calf serum, antibiotics (Gibco, Grand Island, NY) (RPMI), and human recombinant interleukin-1β (IL-1β) (100 ng/ml) (R&D systems Inc., Minneapolis, MN) overnight (12 h) for equilibration at 37°C with 5% CO_2 and 95% humidity. Negative control samples were cultured in the same media without supplementation with IL-1β.

TREATMENT

The following morning, three different concentrations (1×, 0.5×, and 0.1×) of two commercially available sources of HA (A: Synvisc [hylan G-F 20]; B: Hyalgan [sodium hyaluronate]) were added to the media. The 1× concentration of HA added to the culture was determined based on the therapeutic volume of HA normally injected into OA knees (2 ml) divided by the average volume of synovial fluid found in a knee joint (7 ml)[19]. The 0.5× and 0.1× concentrations are in relation to the 1× concentration as summarized in Table I for the four study groups. Negative control samples (without IL-1β), representative of normal articular cartilage and synovium, and positive control samples (with IL-1β), representative of osteoarthritic articular cartilage and synovium, did not receive HA supplementation.

The plates were incubated at 37°C with 5% CO_2 and 95% humidity for 24 h. The HA-containing media were collected and all samples, except the negative controls, were cultured with media supplemented with IL-1β as described above. The media were collected and refreshed every 3 days. Collected liquid media (representative of joint fluid) were stored at −20°C for subsequent evaluation of concentrations of glycosaminoglycan (GAG), matrix metalloproteinase (MMP)-3, prostaglandin E_2 (PGE_2), and nitric oxide (NO).

Cartilage and synovium explants were collected on days 3, 6, and 20. On the days of harvest, cartilage explants were

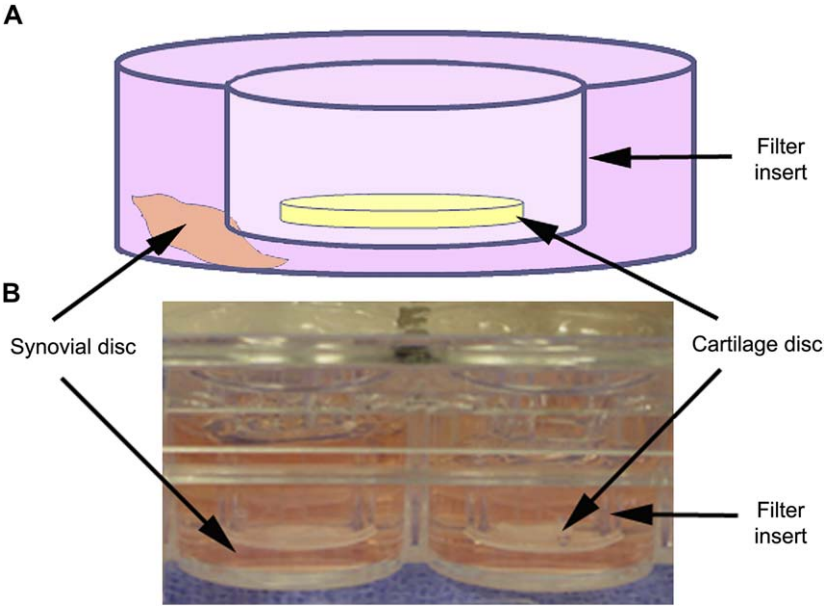

Fig. 1. Schematic diagram (A) and photograph (B) of co-culture model.

Table I
Sample groups

Group names	Treatments
Negative control (untreated group)	RPMI (media alone)
Positive control (IL-1β treated group)	RPMI + 100 ng/ml IL-1β
A3: Synvisc (3)	1× Synvisc in 100 ml RPMI + IL
A2: Synvisc (2)	0.5× Synvisc in 100 ml RPMI + IL
A1: Synvisc (1)	0.1× Synvisc in 100 ml RPMI + IL
B3: Hyalgan (3)	1× Hyalgan in 100 ml RPMI + IL
B2: Hyalgan (2)	0.5× Hyalgan in 100 ml RPMI + IL
B1: Hyalgan (1)	0.1× Hyalgan in 100 ml RPMI + IL

divided into two portions. One portion was weighed (wet weight) and stored at −20°C for subsequent evaluation of water content and matrix contents. The other portion was used for histological analysis.

WATER CONTENT

Harvested cartilage samples were weighed (wet weight), lyophilized, and then weighed (dry weight) again. Water content was calculated for each sample as follows:

$$\text{Water content}\,(\%) = [(\text{Wet weight} - \text{Dry weight})/\text{Wet weight}] \times 100.$$

GAG ANALYSIS

Total sulfated GAG content of liquid media (as an indication of catabolism) and cartilage explants (as an indication of matrix integrity) was quantified using the 1-9-dimethylmethylene blue (DMMB) spectrophotometric analysis[20]. Explant samples were digested in 1.5 ml of digestion solution containing 0.5 mg/ml papain (14 U/mg; Sigma Chemical Co., St. Louis, MO), 20 mM sodium phosphate buffer (pH 6.8) and 1 mM EDTA at 60°C overnight. A 10 μl aliquot of liquid media or digest solution was mixed with 240 μl of DMMB solution and absorbance read at 525 nm using a spectrophotometer (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Bovine tracheal chondroitin sulfate A was used to construct a standard curve to determine the GAG concentration of each sample. GAG concentrations in the media are reported in μg GAG. Explant tissue GAG concentrations were standardized to tissue dry weight and reported as μg GAG/mg dry weight.

PGE_2 AND MMP-3 ANALYSES

Total PGE_2 and MMP-3 content in conditioned media was determined by enzyme immunoassay systems (Amersham International, PLC, Buckinghamshire, England) according to the manufacturer's protocol. PGE_2 concentrations are reported in pg/ml and MMP-3 concentrations are reported in ng/ml.

NO ANALYSIS

Media NO concentration was determined using the spectrophotometric Griess assay (Promega, Madison, WI) according to the manufacturer's protocol[21]. Nitrite (NO_2^-) concentrations in the conditioned media were measured. Culture media (50 μl) were incubated with 50 μl of 1% sulfanilamide at room temperature for 5 min, then 50 μl of 0.1% *N*-naphylethylenediamine dihydrochloride in distilled water. Sample concentrations were determined by measuring the absorbance at 520 nm (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Known concentrations of sodium nitrite were used to construct a standard curve. NO concentrations are reported in μMol.

HISTOLOGY ASSESSMENT

After routine histologic processing, 5-μm cartilage sections were stained with hematoxylin and eosin (H&E) and toluidine blue. Sections were evaluated in triplicate by one investigator (JLC) who was unaware of sample number or treatment group. Sections were evaluated for histologic characteristics of viability, cell morphology, and PG staining. Subjective histologic evaluation was recorded for each group at each time point.

STATISTICAL ANALYSIS

Sample size for this study was determined by analysis of variance (ANOVA) sample size analysis by inputting GAG content as the primary outcome variable and the desired

Table II
Summary of results

	GAG media (μg)			GAG tissue (μg/mg dry wt.)		
	3	6	20	3	6	20
Positive control (IL-1β treated group)	75.1 ± 67.9	70.0 ± 89.2	6.91 ± 5.37	63.9 ± 42.6	92.8 ± 51.3	17.8 ± 12.7
Negative control (untreated group)	115 ± 59.4	119 ± 100	2.88 ± 2.22	73.2 ± 15.9	63.4 ± 30.2	28.7 ± 19.6
Sodium hyaluronate						
0.1×	171 ± 98.3	126 ± 36.4	1.26 ± 1.38	59.8 ± 59.3	47.9 ± 27.3	80.0 ± 47.6
0.5×	277 ± 58.2	229 ± 105	1.40 ± 0.521	218 ± 90.0	33.0 ± 20.0	132 ± 129
1×	163 ± 116	255 ± 106	4.33 ± 6.85	240 ± 154	76.4 ± 55.6	90.2 ± 78.2
Hylan G-F 20						
0.1×	8.31 ± 13.1	85.6 ± 73.1	6.07 ± 10.1	222 ± 83.3	36.3 ± 19.2	29.8 ± 16.7
0.5×	5.34 ± 10.7	68.1 ± 115	2.49 ± 3.17	131 ± 92.3	69.3 ± 42.9	42.4 ± 15.7
1×	28.5 ± 12.0	88.9 ± 102	16.3 ± 11.2	230 ± 107	40.6 ± 26.3	34.0 ± 5.73

power of 0.8 and alpha = 0.05. Based on mean ± S.E.M. values for GAG content from previous studies using *in vitro* explant culture models as data for this analysis, a sample size of $n = 4$ per treatment group was required for the desired power for this study.

All statistical analyses were performed using SigmaStat® (Jandel Scientific, San Rafael, CA). Data from all samples within each group were combined, and medians and means ± S.E.M. were determined for each. Comparisons were made using a *t* test or one-way ANOVA for continuous data. Where significant differences among groups were detected, an all-pairwise multiple comparison procedure (Tukey test) was performed to determine which groups were significantly different from one another. A Kruskal–Wallis one-way ANOVA on ranks was used to compare categorical data. A Spearman rank correlation test was used for determining if significant correlations among the data existed. Significance was set at $P < 0.05$.

Results

All results are summarized in Table II. Table III summarizes the effects of HA treatment on each of the tested variables.

GAG CONTENT AND LOSS

All groups contained measurable concentrations of GAG at all collection times. Although the differences were not statistically significant, *GAG content in cartilage explants* was higher in both HA groups (hylan G-F 20 and sodium hyaluronate) at the beginning (day 3) [Fig. 2(A)] and at the conclusion of the study (day 20) [Fig. 2(B)] compared to the IL-1β-treated (positive control) group. There were no significant differences in GAG content between the two HA groups. No significant differences were noted among the various concentrations. The untreated (negative control) group had more GAG content at the conclusion of the study compared to the IL-1β-treated group.

GAG concentrations in the liquid media were also measurable at all collection times. The GAG content of the media was significantly ($P < 0.05$) lower in the Synvisc group than all other groups on day 3 [Fig. 2(C)]. No significant differences among the concentrations within this group were seen. On day 9, all concentrations in the Hyalgan group had significantly ($P < 0.05$) higher GAG concentrations in the media compared to the untreated group. On day 12, media from the 1× and 0.5× Hyalgan groups had significantly ($P < 0.05$) higher GAG concentrations than the positive and negative controls. However, the Hyalgan group demonstrated a trend of progressively less GAG release late in the study period (after day 15), with the lowest media concentrations of all groups by the end of the study (day 20).

MMP-3 CONCENTRATIONS

The IL-1β-treated group had higher MMP-3 concentrations compared with the untreated group on days 3, 9, 12, 15, and 20. The addition of Synvisc did not decrease the MMP-3 concentrations at any point in the study. On the other hand, MMP-3 concentrations in the liquid media were significantly ($P < 0.05$) lower among the 1× and 0.5× Hyalgan groups on day 20 compared to the IL-1β-treated group [Fig. 2(D)]. No other statistically significant differences among groups or within groups were noted.

PGE_2 CONCENTRATIONS

The PGE_2 concentrations were measurable in all groups at all collection times. On day 3, PGE_2 concentrations were significantly ($P < 0.05$) higher in the IL-1β-treated group compared to the untreated group, all concentrations of the Hyalgan group, and the 0.1× and 1× concentrations of the Synvisc group [Fig. 2(E)]. In general, PGE_2 concentrations were highest at the collection period immediately following IL-1β exposure. Also, the HA groups and IL-1 treated group had more PGE_2 production than the untreated group throughout the study. The highest concentrations of PGE_2 were detected in groups that had been exposed to IL-1β during the first 6 days of culture.

NO CONCENTRATIONS

NO concentrations were measurable in all groups at all collection times. The overall trend seen was that both HA therapy groups had less NO production than the IL-1 β-treated or untreated groups throughout the study period. This observation was best illustrated on days 12 [Fig. 2(F)] and 15, when the NO concentrations were significantly ($P < 0.05$) higher in both the IL-1β-treated and untreated groups compared to all concentrations of both HA groups, except in the 0.1× Synvisc group on day 15. This trend continued through the last day of collection (day 20) with the NO

Table II
(continued)

MMP-3 (ng/ml)			PGE2 (pg/ml)			NO (μMol)		
Day								
3	6	20	3	6	20	3	6	20
1.177 ± 0.181	0.787 ± 1.573	0.482 ± 0.230	172,863 ± 39,214	97,114 ± 59,230	4226 ± 1992	2726 ± 447	1267 ± 568	636 ± 68.8
0.105 ± 2.189	1.043 ± 2.086	0.088 ± 0.067	31,683 ± 32,542	7636 ± 8194	2325 ± 271	1546 ± 585	1437 ± 772	673 ± 168
0	5.991 ± 3.821	1.262 ± 2.524	76,555 ± 70,040	98,201 ± 114,791	4812 ± 3088	2580 ± 467	1304 ± 290	276 ± 51.6
0	8.000 ± 10.30	0	51,397 ± 27,643	158,400 ± 123,057	5826 ± 5306	2605 ± 983	1287 ± 431	302 ± 196
0	3.438 ± 2.703	0	39,119 ± 31,202	182,615 ± 188,548	6482 ± 6941	2653 ± 903	1873 ± 1068	460 ± 221
2.117 ± 1.804	5.592 ± 5.451	0.633 ± 0.448	57,928 ± 46,864	67,769 ± 9822	5053 ± 4702	2322 ± 893	1056 ± 246	398 ± 119
0	8.521 ± 4.966	1.289 ± 2.031	130,061 ± 44,374	94,962 ± 24,501	4883 ± 4667	3641 ± 1457	1348 ± 728	329 ± 135
1.196 ± 2.393	1.935 ± 1.604	0.463 ± 0.498	79,133 ± 36,253	108,581 ± 44,859	3887 ± 5004	1548 ± 510	1504 ± 503	268 ± 71.0

Table III
Effect of HA therapy on GAG content in tissue, GAG content of the liquid media, PGE_2 concentrations, NO concentrations, and MMP-3 concentrations

	GAG tissue	GAG media	PGE_2	NO	MMP-3
Synvisc (hylan G-F 20)	↑ than IL-1β-treated group early (day 3) and late (day 20)	↓ than IL-1β-treated group early	↓ than IL-1β-treated group early (day 3)	↓ than IL-1β-treated group throughout study	No effect
Hyalgan (sodium hyaluronate)	↑ than IL-1β-treated group early (day 3) and late (day 20)	↑ than IL-1β-treated group early, later ↓ (after day 15)	↓ than IL-1β-treated group early (day 3)	↓ than IL-1β-treated group throughout study	↓ than IL-1β-treated group late (day 20)

concentrations significantly ($P < 0.05$) higher in the untreated group than both HA groups, and the OA group NO concentrations significantly ($P < 0.05$) higher than the 0.1× Hyalgan group and the 0.5× and 1× Synvisc groups. NO concentrations were not significantly different among the untreated and IL-1β-treated groups at any time point.

HISTOLOGIC EVALUATION

Subjectively, histologic assessment of matrix staining corresponded well to GAG content in the explants. Day 3 explants had the most intense PG (T-blue) staining, and intensity of staining diminished in all groups over time. Subjective differences in PG staining were seen among groups at each time point evaluated. On day 3, the most intense staining was noted in the 0.5× and 1× Hyalgan groups and all three Synvisc groups. On day 6, the most intense staining was seen in the 0.5× and 1× Hyalgan and 0.5× and 1× Synvisc groups. In addition, the untreated group had subjectively more intense staining than the IL-1β-treated group. On day 20, all three Hyalgan groups had more intense staining than all other groups, and all three Synvisc groups and the untreated group had more intense staining than the IL-1β-treated group (Fig. 3). Subjectively,

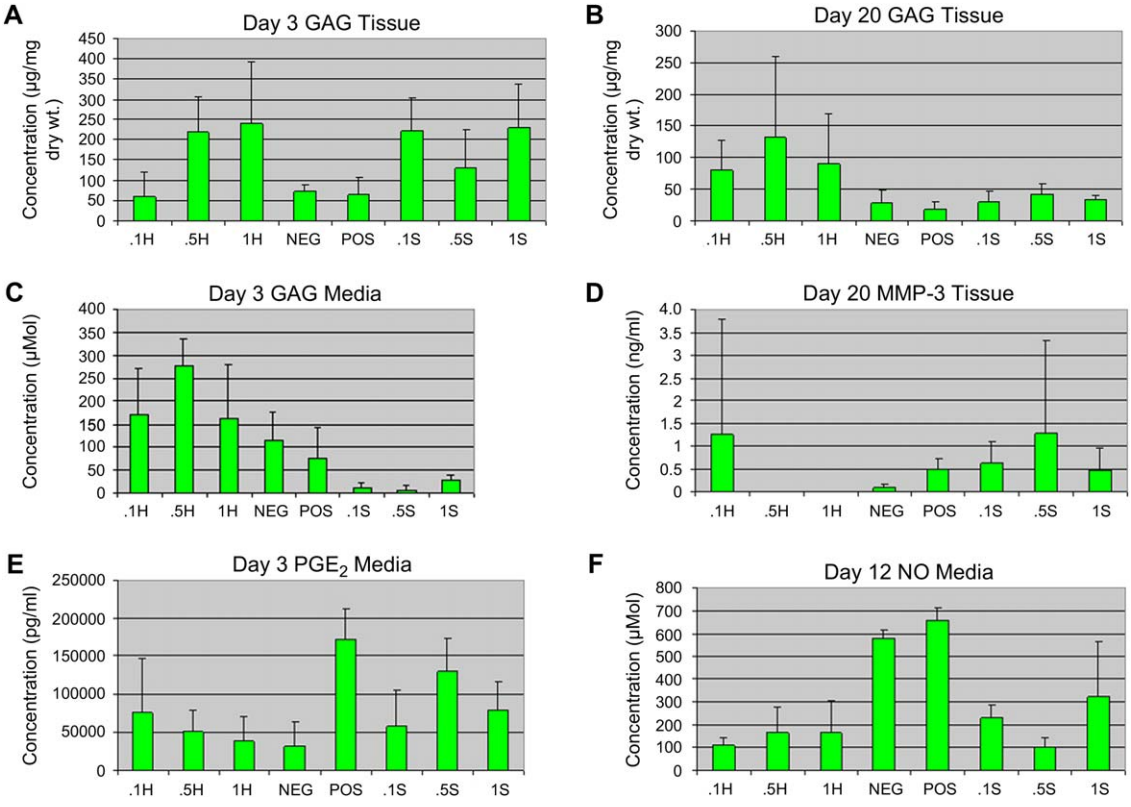

Fig. 2. (A) Mean ± SD concentrations of GAG in cartilage explants on day 3. GAG content in cartilage explants was higher in both HA groups (hylan G-F 20 and sodium hyaluronate) compared to the IL-1β treated group after 3 days. (B) Mean ± SD concentrations of GAG in cartilage explants on day 20. GAG content in cartilage explants was higher in both HA groups compared to the IL-1β treated group at the conclusion of the study. (C) Mean ± SD concentrations of GAG in liquid media on day 3. GAG content of media was significantly ($P < 0.05$) lower in the Synvisc group than all other groups on day 3. (D) Mean ± SD concentrations of MMP-3 in liquid media on day 20. MMP-3 concentrations were significantly ($P < 0.05$) lower among the 1× and 0.5× Hyalgan groups compared to the IL-1β treated group. (E) Mean ± SD concentrations of PGE_2 in liquid media on day 3. PGE_2 concentrations were significantly ($P < 0.05$) higher in the IL-1β treated group compared to the untreated group, all concentrations of the Hyalgan group, and the 0.1× and 1× concentrations of the Synvisc group. (F) Mean ± SD concentrations of NO in liquid media on day 12. NO concentrations were significantly ($P < 0.05$) higher in both the IL-1β treated and untreated groups compared to all concentrations of both HA groups.

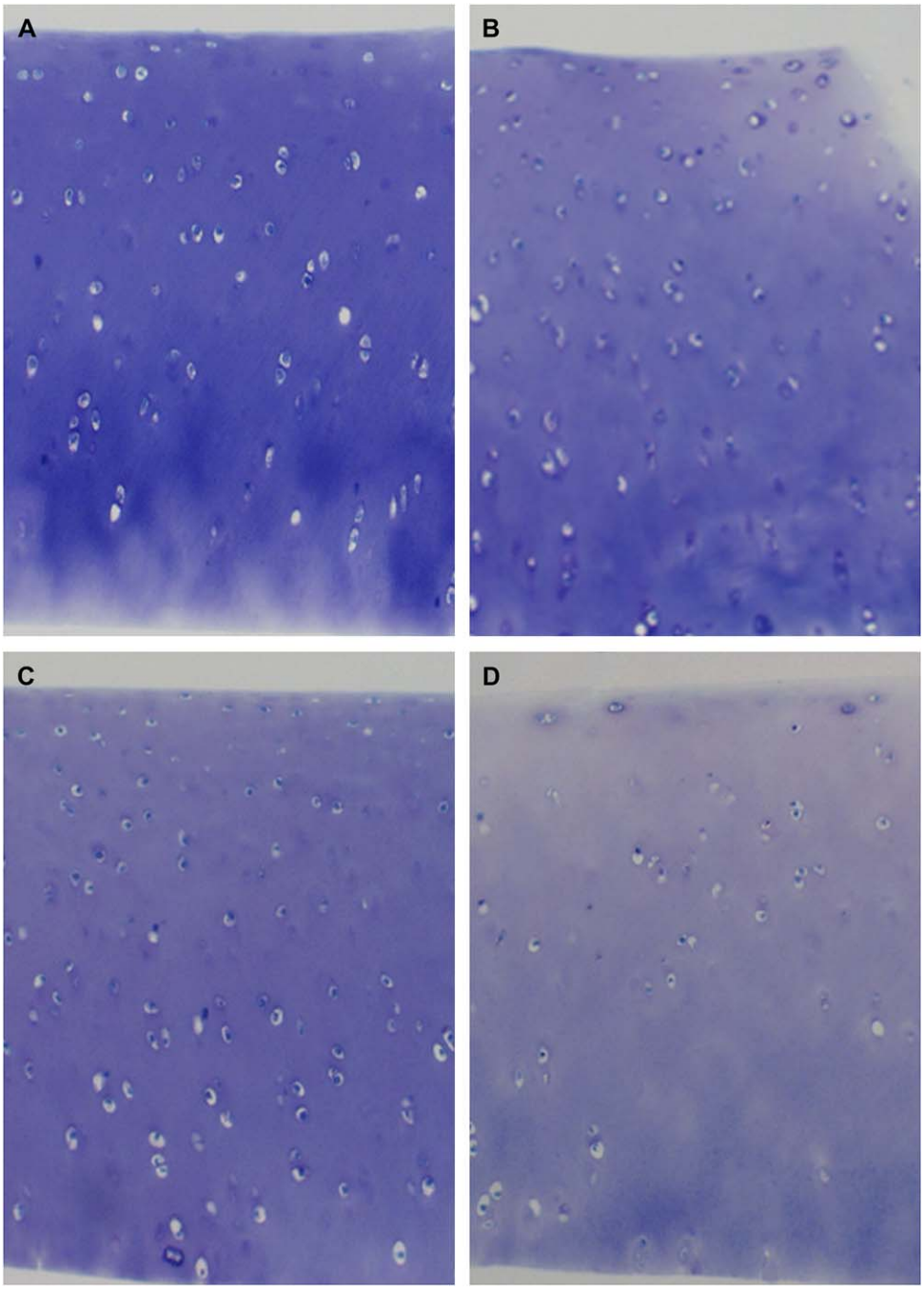

Fig. 3. Photomicrographs showing appearance of cartilage explants from treatment groups after 20 days in co-culture. Subjectively, the Hyalgan groups (A) had more intense staining than all other groups, and the Synvisc groups (B) and the untreated group (C) had more intense staining than the IL-1β treated group (D) (Toluidine blue stain, original magnification 10×.)

no differences in histologic characteristics of cell density or morphology were noted among any groups at any time point.

Discussion

Recently, we have reported the use of a co-culture model for the *in vitro* study of OA[16–18]. In order to validate this model for application to spontaneously occurring OA, we have compared articular cartilage and synovial tissues from dogs that had no evidence of OA and dogs that had OA to cartilage and synovial explants co-cultured in 100 ng/ml of IL-1β (Cook *et al.* 2005, unpublished data in concurrent review). This co-culture model allows for in-depth evaluations of cartilage and synovium, and can be used to create a relatively comprehensive picture of spontaneously occurring OA in dogs. The present study sought to employ this co-culture model to investigate the mechanism of HA therapy in OA. Intra-articular HA is indicated for the treatment of pain in OA of the knee[23]. There are several

commercially available forms of HA, differing in regard to treatment regimens, total dosing, and average molecular weights[10,23]. Hylan G-F 20 has an average molecular weight (MW) of 6 million Da, whereas sodium hyaluronate's average MW is between 0.5 and 0.8 million Da. Of note, the average MW of healthy adult synovial fluid is between 4 and 5 million Da.

HA preparations have been reported to improve symptoms and rating scores and decrease the use of nonsteroidal anti-inflammatory medications in patients with OA. The majority of the evidence suggests that intra-articular administration of HA improves symptoms of OA in selected patients and has few side effects[24–28].

As mentioned above, it is unlikely that the sustained beneficial effects of HA therapy can be accounted for only by a temporary restoration of the synovial fluid lubrication and viscoelasticity[6]. It is more plausible that HA therapy has disease-modifying biological activity and an impact on OA progression. There are four potential mechanisms of action of HA described in the literature that could account for the beneficial effects seen clinically.

The first mechanism is *restoration of elastic and viscous properties* of the synovial fluid. Because our model does not include assessment of HA molecular weight, concentration, or evaluation of media viscoelastic properties, this study was not able to investigate this potential mechanism of action. In an *in vitro* model, using media as a representation of synovial fluid, data regarding the viscoelastic properties of culture media would be, at best, a poor extrapolation of the clinical situation. Therefore, new studies in our laboratory are aimed at addressing the effects of HA on viscous and elastic properties using *in vivo* models.

The second potential mechanism may be derived from the *biosynthetic-chondroprotective effects* of exogenous hyaluronans on cells. Hyaluronans can induce the endogenous synthesis of HA by synovial cells, stimulate chondrocyte proliferation, and inhibit cartilage degradation (chondroprotection)[6,29–33].

In the present study, the elevated GAG content in the HA-treated cartilage tissue at the conclusion of the study compared to the IL-1β-treated group supports a biosynthetic-chondroprotective mechanism. These results are consistent with numerous prior reports in animal models of OA that suggest that hyaluronan therapy has chondroprotective effects[32,34,35]. Larsen *et al.* confirmed that chondrocyte injury and matrix degeneration in cartilage explants subjected to IL-1, degradative enzymes, and oxygen-derived free radicals were reduced by HA treatment[36]. Furthermore, enhanced PG synthesis has been demonstrated[37,38].

Higher concentrations of GAG in the liquid media (representative of joint fluid) may either indicate PG degradation in the cartilage explants and subsequent loss of degraded PG (GAG) to the liquid medium, or release of newly synthesized PGs.

We observed several trends regarding GAG liquid media concentrations: (1) the Synvisc group had less GAG in the media than the other groups early on; (2) the Hyalgan group had more GAG release than all other groups early on (up to day 15); and (3) the Hyalgan group demonstrated a trend of progressively less GAG release late in the study period (after day 15), with the lowest media concentrations of all groups by the end of the study (day 20).

The decreased concentration of GAG in the media in the hylan G-F 20 treatment group coupled with tissue GAG concentrations similar to the untreated group implies decreased GAG degradation (chondroprotection). Samples treated with sodium hyaluronate did not have lower media GAG concentrations until late in the study period. This may be due to a delayed therapeutic effect with this specific form of HA.

Enzymatic activity plays an important role in OA. Takahashi and colleagues found that HA therapy down-regulated MMP-3 expression in the synovium of rabbits with mild/early OA[32]. In the present study, as the concentration of GAG in the media of Hyalgan treatment groups decreased, so did the concentrations of MMP-3 in media. This implies that the decreased release of GAG seen with Hyalgan, may be due in part to decreased expression and activity of degradative enzymes. However, significant reductions in media MMP-3 concentrations were not observed for Synvisc samples, indicating that hylan G-F 20 treatment did not affect the expression of this degradative enzyme (MMP-3) in this study. Future studies are required to definitively determine if chondroprotective effects of HA are the result of decreased degradation, increased synthesis, or both.

The *anti-inflammatory effects* that have been observed with HA suggest another potential mechanism. HA therapy has been associated with decreases in inflammatory cell count in the synovial fluid, modulating cytokine expression, and reducing superoxide radicals[23]. NO and PGE_2 are markers of inflammation associated with chondrodegradation. Takahashi *et al.* showed that NO production by the meniscus and synovium was reduced by the administration of HA[39]. HA has also been shown to suppress synovial cell release of PGE_2 secondary to IL-1α activation[40,41]. In the current study, PGE_2 and NO concentrations were increased after IL-1β stimulation (IL-1β-treated group) compared to the HA or untreated groups. As these are both inflammatory markers, HA therapy appears to modulate the inflammatory process seen in OA.

PGE_2 concentrations were highest at the collection period immediately following IL-1β exposure, results consistent with prior work in our laboratory[22]. The decline in PGE_2 concentrations appeared to coincide with the loss of cell viability after exposure to IL-1β. The cell loss may have resulted in less PGE_2 production secondary to the smaller number of cells present to respond. This theory appears to be supported by the correlations between declining PGE_2 concentrations and GAG content and loss.

Finally, there is evidence to support an *analgesic effect* of hyaluronan[42,43].

Pozo *et al.* reported that intra-articular HA reduced nerve impulse activity in nociceptive afferent fibers in a cat model of acute arthritis[44].

There are several limitations to our study. Our *in vitro* model does not account for load, blood flow, joint function, or pain. This limited us to investigating only two of the four potential mechanisms outlined in the literature. The concentrations of HA used were based on the best available data. No significant differences were noted among the concentrations in any of the variables tested. Thus, we are confident that the range of concentrations used was representative of *in vivo* conditions. Based on the broad spectrum of potential mechanisms investigated, we were limited in the number of assessments we were able to perform. Therefore, we could not make definitive conclusions regarding the clinical effects of HA or differences among HA products. Ongoing studies in our laboratory are aimed at further defining the mechanisms of action of HA therapy from molecular to clinical levels.

Conclusion

This study supports two potential mechanisms of action of viscosupplementation. The first is a biosynthetic-chondroprotective mechanism, with possibly a delay in

onset depending on the form of HA. The second is an anti-inflammatory mechanism of action. In addition to providing mechanistic evidence, the outcomes in this study further validate the co-culture model for the investigation of OA. Insight into the mechanisms and biological effects of hyaluronans may serve as a guide toward future therapy.

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