Effects of intra-articular hyaluronic acid on ex vivo responses by third carpal bone cartilage from horses with experimentally induced synovitis

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Abstract
We studied the extent to which intra-articular hyaluronic acid (HA) administered to horses with experimentally induced synovitis in the third middle carpal joint altered cartilage proteoglycans (PGs) and the biomechanical properties of the third carpal bone. Intra-articular HA (Hylartin-V®) was administered to the middle carpal joints of healthy horses before and after the induction of synovitis by Escherichia coli lipopolysaccharide (LPS). Two groups of horses received intra-articular HA only, with evaluation 2 and 6 weeks later (group 2 and group 4). Two other groups (group 1 and group 3) received LPS followed by intra-articular HA and evaluation 2 and 6 weeks later. Ex vivo third carpal bone cartilage PG synthesis was measured by 35SO4 incorporation and third carpal bone cartilage PG content was determined by an assay for glucuronic acid. Biomechanical properties were measured by indentation testing. At the 2-week evaluation, HA did not ablate the quantitative reduction in PG synthesis and PG content in animals with experimental synovitis. However, by 6 weeks, there was a significant rebound effect in that PG synthesis and PG content were elevated in horses with experimental synovitis that had received intra-articular HA, compared with horses that had received HA only for that period of time. There were also alterations in the gel filtration profiles of newly synthesized PGs on Sepharose CL-2B among the groups. However, there were no de novo PG species associated with any of the treatments. Furthermore, we found that the biomechanical properties of third carpal bone cartilage were not altered in any of the groups. These studies showed that intra-articular HA administered to horses with experimentally induced synovitis did not provide short-term (i.e. 2-week) benefit to third carpal bone cartilage PG synthesis. However, a long-term benefit of intra-articular HA may occur after the acute effects of synovitis on PG synthesis and PG content subside.

Keywords: hyaluronic acid; equine bone cartilage; synovitis

Introduction
Hyaluronic acid (HA) is a component of both synovial fluid and the extracellular matrix of articular cartilage. Synovial fluid HA is thought to function by decreasing the frictional forces generated between synovial membrane surfaces during joint movement1,2. Synovial membrane Type B cells and chondrocytes synthesize HA by disaccharide oligomerization3,4. While HA...
contributes to synovial fluid viscosity, the role of articular HA is quite different from that of synovial fluid HA. Cartilage HA provides the molecular mechanism whereby sulfated proteoglycans (PGs) (i.e. aggregan and versican) are immobilized in the cartilage extracellular matrix via non-covalent association between PG and HA (i.e. PG aggregates). PG aggregates increase the viscoelastic properties of cartilage mainly by affecting compressive stiffness, which is critical to cartilage homeostasis. A ‘free’ form of HA synthesized by cultured articular chondrocytes appears to subserve cartilage HA that forms complexes with PG and, as chondrocytes possess HA receptors, they provide a cellular coupling mechanism for HA and/or PG aggregate binding to chondrocytes. HA receptor occupancy may also potentially modulate chondrocyte responses to synovial fluid HA or exogenous HA.

Intra-articular HA supplementation (i.e. viscosupplementation) may enhance the viscoelastic properties of synovial fluid which are diminished in inflammatory arthropathy. This serves as a rationale for restoring the rheological properties of synovial fluid via viscosupplementation. Viscosupplementation may also provide a diffusion barrier to potentially deleterious inflammatory mediators and/or enzymes (released by activated macrophages and/or synoviocytes) which possess the capacity to attach to the articular cartilage surface. A putative role of HA in regulating cartilage homeostasis may be independent of any changes in the rheological properties of synovial fluid after intra-articular HA administration.

Intra-articular HA has been a widely accepted treatment for acute traumatic synovitis that occurs regularly in equine veterinary practice devoted to the management of performance horses. HA has also been studied for efficacy in treating acute synovitis episodes associated with equine osteoarthritis. However, Caron et al. reported in a comparison study of polysulfated glycosaminoglycan (PSGAG) versus HA that PSGAG was more effective than sodium hyaluronate for the treatment of subacute degenerative joint disease but HA was more effective for treating idiopathic joint effusion and acute synovitis.

The majority of veterinary studies as well as human clinical trials that have assessed HA as an arthritis therapy have relied mainly on parameters of clinical improvement, such as decreased joint pain and increased range of motion, to determine efficacy. These clinical outcome measures have long been criticized as being too subjective. Since HA was approved for use in equine veterinary practice, few, if any experimental studies have focused on objective biochemical and biomechanical measurements to determine the efficacy of HA. In studies that showed clinical efficacy attributed to HA, the results of clinical improvement have not been adequately supported by objective surrogate measurements of disease activity. For example, only recently have experimental studies measured HA effects on chondrocyte metabolism. However, these analyses have not been studied in parallel with any clinical efficacy studies of HA on cartilage metabolism in vivo.

In this regard, in vitro studies have shed light on how HA might alter cartilage metabolism when administered in vivo. These results provide a compelling rationale for assessing the clinical usefulness of HA in treating inflammation, as in vitro studies have demonstrated that HA suppressed PG degradation and release of PG from the matrix of cartilage explants and from chondrocyte cultures. Recent studies have also shown that lipopolysaccharide (LPS) - employed to induce equine inflammatory synovitis and joint inflammation in guinea pigs - upregulated matrix metalloproteinase (MMP) synthesis. In addition, experimental animal models of osteoarthritis and cruciate ligament healing have shown that HA may delay cartilage changes by reducing PG degradation and structural alterations in PG following intra-articular corticosteroid administered to normal animal joints.

This laboratory has previously reported that, compared with sham-treated horses, PG synthesis was increased and Poisson's ratio (a measure of tissue compressibility) was lowered 2 weeks after induction of experimental synovitis. However, prior to the present study, the capacity for intra-articular HA to alter PG synthesis, PG content or cartilage biomechanical properties in the inflamed equine joint induced experimentally by Escherichia coli LPS had not been studied. The effect of intravenous HA on chondrocyte metabolism by equine cartilage after induction of inflammation by osteochondral fragmentation was, however, previously reported by Kawcak et al., but to our knowledge the design of the present study is one of a only a few protocols employed to examine efficacy of intra-articular HA administration in synovitis with ex vivo assessment of cartilage metabolism and its biomechanical properties.
Materials and methods

Animals
The study design and experimental procedures were approved by The Ohio State University Laboratory Animal Care and Use Committee. Eight healthy adult horses were selected for study (three to nine years old, body weight 360–500 kg) on the basis of normal clinical, orthopaedic and radiographic examination as well as normal synovial fluid analysis. Horses were individually housed in 12 ft × 12 ft box stalls. The physical activity of the horses was standardized on a high-speed treadmill (Swedish Sato, Uppsala, Sweden) for 20 min once weekly at 4 m s⁻¹ for 4 weeks prior to initiating the study. This protocol was also designed to standardize the level of cartilage metabolism among the horses, as previously described³⁷.

E. coli LPS-induced synovitis
E. coli LPS was employed to create a mild to moderate acute synovitis in the middle carpal joint as previously reported³¹. Carpi were aseptically prepared for intra-articular LPS administration. LPS (serotype B:055; Difco Laboratories, Detroit, MI) (0.125 ng) in phosphate-buffered saline was injected bilaterally into the middle carpal joint every 48 h for 1 week. Four treatments were employed to maintain synovial inflammation³¹.

Study design
The study design was modified from one previously reported by Palmer et al.³⁷. Sixteen carpi dissected from eight horses were studied in four groups of animals (Table 1).

- Group 1: Synovitis induced with E. coli LPS at time zero followed by intra-articular HA (20 mg) (Hylaritin-V; Pharmacia/Upjohn AB, Sweden and Kalama-zoo, MI) 1 week later with evaluation at 2 weeks.
- Group 2: Sham treatment as previously described³⁷; intra-articular HA with evaluation at 2 weeks.
- Group 3: Synovitis at time zero, followed by intra-articular HA 1 week later. These animals were evaluated at 6 weeks.
- Group 4: Sham treatment; intra-articular HA with evaluation at 6 weeks.

Physical examination
Prior to sacrifice, horses were evaluated for synovitis by the presence or absence of joint effusion and pain on palpation as previously described³¹, as well as the degree of lameness by employing a lameness scoring system⁴⁰.

Tissue sampling and clinical analysis
Horses were euthanized with an overdose of sodium pentobarbital. Mid-carpal joints were aseptically opened and third carpal bone articular cartilage was assessed for gross anatomical abnormalities. All clinical tests were performed in the clinical pathology laboratory at The Ohio State University. Joint fluid leucocyte counts were performed using an automated Coulter counter (Beckman, Fullerton, CA). Cell sediments were displayed on glass slides, stained with DiffQuik and at least 100 cells counted in a haemocytometer (Fisher Scientific, Hampton, NH) by a board-certified veterinary clinical pathologist. Cartilage was collected from the dorsal and palmar aspects of the central aspect of the radial facet for PG analysis and from the medial aspect of the radial facets for mechanical studies as previously described³⁷,⁴¹, and stored at −80°C.

Specimens collected for PG analysis (~2 mm in diameter) were obtained using a power drill and core biopsy instrument⁴². The biopsy sites were continuously irrigated with Ringer’s lactate solution (Baxter Healthcare Corp., Deerfield, MI) during tissue harvesting to minimize thermal necrosis⁴². Cartilage was immediately immersed in separate sterile vials containing chilled Dulbecco’s modified Eagle’s medium (DMEM) (Gibco/BRL, Gaithersburg, MD).

Synthesis and characterization of equine third carpal cartilage PG
Equine cartilage explants and PG extraction
The analysis of equine third carpal bone cartilage sulfated PG synthesis using ex vivo radiolabelling with

<table>
<thead>
<tr>
<th>Week</th>
<th>Group 1 (n = 4)</th>
<th>Group 2 (n = 4)</th>
<th>Group 3 (n = 4)</th>
<th>Group 4 (n = 4)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Synovitis</td>
<td>Sham treated</td>
<td>Synovitis</td>
<td>Sham treated</td>
</tr>
<tr>
<td>1</td>
<td>HA</td>
<td>HA</td>
<td>HA</td>
<td>HA</td>
</tr>
<tr>
<td>2</td>
<td>Termination</td>
<td>Termination</td>
<td>No additional HA therapy</td>
<td>No additional HA therapy</td>
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<tr>
<td>3</td>
<td></td>
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<td></td>
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<td>6</td>
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HA – hyaluronic acid. The physical activity of all horses was standardized on a high-speed treadmill for 20 min once weekly at 4 m s⁻¹ for 4 weeks prior to random assignment to group.


$^{35}$SO$_4$ has been described in detail previously. This anatomical site was chosen for PG analysis because it is one of the most commonly affected bones with this type of inflammatory injury in the performance horse. In this study, cartilage explants were incubated for 18 h at 37°C in an atmosphere of 5% CO$_2$/95% air in DMEM containing foetal bovine serum (10% v/v) and Na$_2$ $^{35}$SO$_4$ (40 μCi ml$^{-1}$). Minced cartilage was extracted overnight at 4°C by constant stirring with 4 M guanidine hydrochloride (GuHCl) 0.1 M sodium acetate buffer (pH 5.8) containing a ‘cocktail’ of protease inhibitors which included 6-aminohexanoic acid (100 mM), disodium ethylenediaminetetraacetic acid (10 mM) and benzamidine (5 mM), designed specifically to prevent PG degradation during 4 M GuHCl extraction. 4 M GuHCl extracts were dialysed overnight against distilled water (molecular weight cut-off, 6000–8000) to remove unincorporated $^{35}$SO$_4$ as well as to promote PG aggregation to any available HA remaining within the PG extract. PG content in the dialysed 4 M GuHCl and PG content remaining in the minced cartilage after 4 M GuHCl extraction were determined by the modified carbazole reaction for glucuronic acid. Cartilage protein content was measured in the same 4 M GuHCl extract by the bicrochoninic acid assay. Glucuronic acid content in the dialysed 4 M GuHCl extract and hydrolysed tissue residue were employed to determine the efficiency of PG extraction as previously described.

$^{35}$SO$_4$ incorporated into newly synthesized PG was determined on an aliquot of the dialysed 4 M GuHCl extract in a liquid scintillation counter (Tri-carb model 3255; Beckman Packard, Palo Alto, CA). The results are expressed as cpm $^{35}$SO$_4$ μg$^{-1}$ protein. The endogenous PG content of the dialysed 4 M GuHCl extract was expressed as μg glucuronic acid μg$^{-1}$ protein. An aliquot of the PG extract was pooled according to site and group, freeze-dried and stored at −80°C prior to Sepharose CL-2B chromatography.

**Sepharose CL-2B chromatography**

The hydrodynamic size of 4 M GuHCl extractable, newly synthesized and endogenous PG was determined by Sepharose CL-2B chromatography. Freeze-dried PG extracts from each group were dissolved in 0.5 M sodium acetate buffer (pH 7.0). Samples were chromatographed on a column (0.9 cm x 100 cm) of Sepharose CL-2B (Pharmacia Fine Chemicals, Piscataway, NJ) with and without prior incubation with exogenous HA (human umbilical cord; Sigma Chemical Co., St Louis, MO) and eluted with 0.5 M sodium acetate buffer as previously described. The radioactivity in each column fraction was measured by liquid scintillation spectroscopy. Since PG peak II was the predominating peak on Sepharose CL-2B chromatography in most of the groups, the eluted fractions from this peak were pooled and freeze-dried.

**Composite agarose/polyacrylamide gel electrophoresis and Western blotting**

Composite agarose/polyacrylamide gel electrophoresis (CAPAGE) was employed to characterize the electrophoretic migration of equine PG as compared with a purified cartilage PG monomer standard. Western blots were used to determine the extent to which equine PG contained the HA-binding region. To create the epitope in the PG monomer HA-binding region recognized by monoclonal antibody (Mab) 1C6 (Developmental Studies Hyridoma Bank, Baltimore, MD), pooled peak II column fractions from group 1 and group 4 dorsal and palmar sites were reduced with 10 mM dithiothreitol in 4 M GuHCl/1 mM Tris (pH 8) for 4 h at 37°C and then alkylated with 80 mM iodoacetate in 4 M GuCl/1 mM Tris (pH 8), as previously described. Samples were electrophoresed on duplicate composite gels; one gel was stained for PG with Toluidine blue-O while the other gel was employed for Western blotting with Mab 1C6 following electrotransfer of PG to Immobilon-P blotting membranes. Reduced and alkylated PG monomer (fraction A1D1) purified from human articular cartilage by caesium chloride density ultracentrifugation was employed as the positive control for Mab 1C6 reactivity.

**Indentation testing**

The biphasic biomechanical properties of the third carpal bone articular cartilage were determined by indentation testing as previously described. In brief, cartilage was loaded through a porous, plane-ended, cylindrical indenter (Fig. 1). The indenter displacement was measured as a function of time using a linear variable displacement transducer. Initially, displacement at the cartilage surface was relatively rapid following the application of constant weight. However, the displacement rate decreased over time reaching essentially a constant value at which time the test was terminated. Typical tests required 25 to 67 min to complete.

Specimens were mounted in the indentation apparatus holder with cyanoacrylate cement and thawed for 1 h at room temperature in 0.15 M NaCl containing proteinase inhibitors as previously described. Twelve sites from each carpus were analysed. These sites were located in the centre of the dorsal aspect of the medial compartment of the radial facet or in the centre of the palmar aspect of the medial compartment of the radial facet.

The aggregate modulus, Poisson’s ratio and permeability were calculated from measurements made on the indentation apparatus. Cartilage creep
recovery was also performed at each site to confirm appropriate indentation testing parameters and tissue health. Cartilage thickness at each site was determined after creep recovery by measuring the displacement of a pin between its contact point at the articular cartilage surface and subchondral bone. Contact with each interface was detected by the change in force on the pin.

Statistical analysis
Groups were compared by repeated-measures mixed analysis of variance (Statistical Analysis System; SAS Institute, Cary, NC) with Bonferroni's correction applied for each site and/or group. This analysis also corrected for variance among specimens obtained from individual horses as previously reported. A \( P \)-value of \(< 0.05\) was considered significant. Specifically,

- Groups 1 and 2 were compared to evaluate the effects of intra-articular HA administered to animals with synovitis and to normal animals after 2 weeks.
- Groups 3 and 4 were compared to evaluate the effects of intra-articular HA administered to animals with synovitis and to normal animals after 6 weeks.
- Groups 1 and 3 were compared to evaluate the effect of time following HA administration to inflamed joints.
- Groups 2 and 4 were compared to evaluate the effect of time following HA administered to normal joints.

Results

Clinical and pathological findings
Intra-articular LPS administered to healthy adult horses caused mild to moderate synovitis in groups 1 and 3. Synovitis was characterized by joint effusion, pain on palpation and grade 1–2 lameness, which commenced 12 h after the initial LPS administration and persisted throughout the LPS injection protocol. However, all clinical signs of inflammation, including leucocyte counts, subsided within 1 week after the last LPS injection and were not altered by intra-articular HA. Horses receiving HA only (i.e. groups 2 and 4) did not show clinical evidence of joint effusion or warmth, nor did they resist palpation or joint flexion at any time. There was no change in body weight or appetite with any treatment.

At necropsy, the synovial membrane, synovial fluid and articular cartilage in each joint appeared grossly normal, excluding the dorsal site in one horse from group 4, which showed a gross cartilage lesion. This site was neither analysed nor included in the statistical analysis.

PG extraction, synthesis and content
PG extraction efficiency was determined by the amount of PG in dialysed 4 M GuHCl extracts and completely hydrolysed cartilage after 4 M GuHCl extraction. Endogenous PG content measured by the carbazole reaction did not differ among the randomized groups (average PG extracted = 47.5%). \( ^{35} \text{SO}_4 \) incorporation and PG content of third carpal joint cartilage from normal horses that received HA were compared with values from horses with synovitis that received HA (Table 2). At the 2-week evaluation, third carpal bone cartilage PG synthesis and PG content were greater from horses that had received intra-articular HA compared with horses with synovitis that received HA (Table 2). However, PG synthesis and PG content values in the latter group were greater (mean, 33 000 cpm \( ^{35} \text{SO}_4 \mu \text{g}^{-1} \text{ protein} \)) than in our previously studied comparable group of horses with untreated synovitis (mean, 11 000 cpm \( ^{35} \text{SO}_4 \mu \text{g}^{-1} \text{ protein} \)).

At the 6-week evaluation, PG synthesis and PG content in horses with synovitis treated with HA were significantly greater than at 2 weeks (Table 2), suggesting a rebound effect in PG synthesis and PG content induced by HA during the 4 weeks after HA was first administered.

PG chromatographic profiles
PG extracts were chromatographed on Sepharose CL-2B to determine the extent to which the hydrodynamic size of newly synthesized PG differed among the four groups. The chromatographic profiles for
group and site essentially overlapped (Figs 2a–2d). All groups exhibited a shoulder of $^{35}$SO$_4$ eluting at or near the column void volume (i.e. fractions 16–19), an early peak (i.e. peak I; fractions 20–39; $K_{av}$ 0.10–0.19) eluting just to the right of the column void volume and a late eluting peak (i.e. peak II; fractions 40–56; $K_{av}$ 0.60–0.73).

The percentage of newly synthesized PG eluting under the shoulder and under each peak of the Sepharose CL-2B chromatogram was determined for each of the four groups by mathematical integration. A distinct distribution profile of newly synthesized PGs emerged from this analysis. Table 3 shows that the dorsal PGs from group 1 (i.e. synovitis + HA, 2 weeks) were reduced in the shoulder ($\Delta$, 36.7), reduced in peak I ($\Delta$, 30) but increased in peak II ($\Delta$, 23.2), compared with group 2 (i.e. HA, 2 weeks). By contrast, the palmar aspect PG distribution in peaks I and II was largely unchanged when the group 1 elution pattern was compared with the elution pattern of group 2. A converse profile of newly synthesized PG emerged when group 3 (i.e. synovitis + HA, 6 weeks) was compared with group 4 (i.e. HA, 6 weeks). Here, the dorsal aspect PG in group 4 was reduced in peak I ($\Delta$, 36.2) and increased in peak II ($\Delta$, 30.4) when compared with group 3 (Table 2). Once again, the palmar aspect PGs distribution was largely unchanged when these two groups were compared. Addition of umbilical cord HA to the PG extract did not alter the elution profile from any group or anatomical site (data not shown).

### Table 2 Effect of HA on newly synthesized and endogenous cartilage PG

<table>
<thead>
<tr>
<th>PG measured</th>
<th>Group 1 (synovitis + HA, 2 weeks)</th>
<th>Group 2 (HA, 2 weeks)</th>
<th>Group 3 (synovitis + HA, 6 weeks)</th>
<th>Group 4 (HA, 6 weeks)</th>
</tr>
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<tbody>
<tr>
<td>Synthesized PG$^a$</td>
<td>3.3 ± 0.5</td>
<td>12.2 ± 3.2</td>
<td>9.5 ± 1.3</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Endogenous PG$^b$</td>
<td>0.28 ± 0.01</td>
<td>0.47 ± 0.05</td>
<td>0.38 ± 0.05</td>
<td>0.28 ± 0.005</td>
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Repeated-measures mixed ANOVA with Bonferroni’s correction:

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<tr>
<th>Synthesized PG</th>
<th>Endogenous PG</th>
<th>$P$-value</th>
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<tbody>
<tr>
<td>1 vs. 2</td>
<td>&lt;0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>3 vs. 4</td>
<td>&lt;0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>1 vs. 3</td>
<td>&lt;0.05</td>
<td>0.05</td>
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<tr>
<td>2 vs. 4</td>
<td>&lt;0.05</td>
<td>0.05</td>
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</table>

$^a$ In units of $^{35}$SO$_4$ cpm x 10$^3$/µg protein (mean ± SEM).

$^b$ In units of µg glucuronic acid µg$^{-1}$ protein (mean ± SEM).

The percentage of newly synthesized PG eluting under the shoulder and under each peak of the Sepharose CL-2B chromatogram was determined for each of the four groups by mathematical integration. A distinct distribution profile of newly synthesized PGs emerged from this analysis. Table 3 shows that the dorsal PGs from group 1 (i.e. synovitis + HA, 2 weeks) were reduced in the shoulder ($\Delta$, 36.7), reduced in peak I ($\Delta$, 30) but increased in peak II ($\Delta$, 23.2), compared with group 2 (i.e. HA, 2 weeks). By contrast, the palmar aspect PG distribution in peaks I and II was largely unchanged when the group 1 elution pattern was compared with the elution pattern of group 2. A converse profile of newly synthesized PG emerged when group 3 (i.e. synovitis + HA, 6 weeks) was compared with group 4 (i.e. HA, 6 weeks). Here, the dorsal aspect PG in group 4 was reduced in peak I ($\Delta$, 36.2) and increased in peak II ($\Delta$, 30.4) when compared with group 3 (Table 2). Once again, the palmar aspect PGs distribution was largely unchanged when these two groups were compared. Addition of umbilical cord HA to the PG extract did not alter the elution profile from any group or anatomical site (data not shown).

### Table 3 Sepharose CL-2B fractionation of of $^{35}$SO$_4$-PG

<table>
<thead>
<tr>
<th></th>
<th>Shoulder (fractions 16–19) (%)</th>
<th>Peak I (fractions 20–39) (%)</th>
<th>Peak II (fractions 40–56) (%)</th>
</tr>
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<tbody>
<tr>
<td>Group 1 (synovitis + HA, 2 weeks)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Palmar</td>
<td>5.3</td>
<td>45.8</td>
<td>48.9</td>
</tr>
<tr>
<td>Dorsal</td>
<td>2.6</td>
<td>49.3</td>
<td>48.1</td>
</tr>
<tr>
<td>Group 2 (HA, 2 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmar</td>
<td>9.3</td>
<td>41.3</td>
<td>49.4</td>
</tr>
<tr>
<td>Dorsal</td>
<td>6.1</td>
<td>31.2</td>
<td>62.7</td>
</tr>
<tr>
<td>Group 3 (synovitis + HA, 6 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmar</td>
<td>4.2</td>
<td>41.2</td>
<td>54.6</td>
</tr>
<tr>
<td>Dorsal</td>
<td>3.4</td>
<td>34.0</td>
<td>62.8</td>
</tr>
<tr>
<td>Group 4 (HA, 6 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmar</td>
<td>2.8</td>
<td>36.8</td>
<td>60.4</td>
</tr>
<tr>
<td>Dorsal</td>
<td>3.6</td>
<td>53.3</td>
<td>43.1</td>
</tr>
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PG – proteoglycan; HA – hyaluronic acid. The percentage of $^{35}$SO$_4$ eluting under the shoulder and two peaks of the chromatograms was determined from Fig. 2 by mathematical integration.
Analysis of PG monomer by CAPAGE: Western blotting with Mab 1C6

Because newly synthesized PG from peak II of the Sepharose CL-2B chromatogram was hydrodynamically smaller than expected for intact PG monomer, CAPAGE was performed to determine the migration position of this PG relative to human cartilage purified PG monomer. A broad band (PGII) that stained with Toluidine blue-O migrated only somewhat faster (Fig. 3A, lanes 1 and 2) than reduced and alkylated human intact PG monomer (Fig. 3A, lane 3), indicative of the hydrodynamically smaller nature of the equine PG on Sepharose CL-2B compared with human PG monomer. However, dorsal and palmar site PG from horses with synovitis that had received HA or from horses receiving HA alone (i.e. groups 1 and 4) migrated to a similar position on the gel (Fig. 3A, lanes 1 and 2).

Furthermore, Western blotting with Mab 1C6 (Fig. 3B) showed that the PG from both palmar and dorsal aspects of third carpal bone cartilage from these groups contained the HA-binding region (Fig. 3B, lane 3) employed human cartilage intact PG monomer (fraction A1D1)5,41.

Indentation testing

There were no significant differences in aggregate modulus, Poisson’s ratio, permeability or cartilage thickness among the four groups, or within dorsal and palmar sites in the four groups (Figs 4a–4d). The average cartilage creep recovery among the groups was 98.7%, indicating uniformity among the groups and sites for indentation testing parameters and tissue health.

Discussion

The results of the present study did not support a salutary effect on quantitative ex vivo third carpal bone cartilage PG synthesis and PG content from a single intra-articular administration of HA to horses with LPS-induced synovitis evaluated after 1 week of HA therapy, although a more significant HA effect was seen when the 2-week results of the present study were compared with our previously reported results from studies of untreated equine synovitis37. At the 6-week evaluation, cartilage PG synthesis and PG content from horses with synovitis that were treated with HA had returned to levels comparable to those seen at 2 weeks in horses receiving only HA. Indeed, the effect of HA at 6 weeks may be especially significant since we previously reported37 that horses with untreated LPS-induced synovitis continued to exhibit reduced PG synthesis at 6 weeks, whereas in the present study with HA an apparent ‘rebound’ occurred. This comparison suggested that HA was beneficial when the 6-week evaluation was considered and that apparent salutary effects of HA were delayed until a period when cartilage recovery responses were apparently more optimal for PG synthesis.

It is possible that the 2-week evaluation may have been compromised by elevated HA clearance from the joint as a result of induced inflammatory-mediated changes in the joint prior to HA administration. Indeed, in vivo canine studies have shown that synovitis increases both protein and HA clearance from the joint52,53. Further, Hylartin-V® administered to horses did not increase the specific viscosity54 (defined at a specific temperature and shear rate) of carpal bone synovial fluid, which was strongly affected by HA concentration. However, even in the absence of inflammation, HA was shown to be removed rapidly from...
the equine joint after its administration. That study found that intra-articular radiolabelled HA bound avidly to synovial membrane and articular cartilage for greater than 48 h, but not for more than 7 days. Thus, normal HA clearance rates combined with the effects of antecedent inflammation may have reduced the effectiveness of HA at 2 weeks. Long-term positive effects of HA on third carpal bone cartilage PG (i.e. at 6 weeks) may have occurred because HA clearance rates were reduced during the 3–6 week period of the study, at which time no clinical evidence of inflammation could be demonstrated.

Suppression of PG synthesis and decreased PG content 2 weeks following the induction of synovitis in horses that received HA (Table 2) suggested that inflammatory cytokines known to be active in acute synovitis may have been responsible for chondrocyte responses at that time. Indeed, previous studies by Kawcak et al. demonstrated a sustained increase in blood flow to the joint and increased levels of interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) in synovial fluid in another model of equine synovitis induced by osteochondral fragments. Additionally, Todhunter et al. provided immunohistochemical evidence that supports IL-1β and TNF-α protein upregulation as well as increased synthesis of TNF receptors p55 and p75 in another LPS-induced model of equine synovitis. Several additional studies support the conclusion that synovitis induced by E. coli LPS augmented MMP synthesis and IL-1 production by macrophages, synoviocytes and chondrocytes, all of which play a significant role in cartilage matrix degradation during inflammation and may have been responsible for a loss in PG content and suppression of PG synthesis. Taken together, these studies as well as other assessments support the view of augmented cartilage degeneration and blunted cartilage repair responses occurring in joints exhibiting markedly increased levels of cytokines and MMPs, which may be the molecular targets for HA therapy.

To determine the extent to which HA altered the pattern of newly synthesized PG in the various study...
groups, dorsal and palmar site cartilage PG species in dialysed 4M GuHCl extracts were resolved on Sepharose CL-2B. A shoulder and two distinct PG peaks of newly synthesized PG emerged from this analysis, indicating that synovitis treated with HA did not result in alterations in the PG hydrodynamic size nor did the elution profiles represent PG aggregates formed by the addition of exogenous HA to the dialysed cartilage extracts. This present chromatographic profile differed, however, from what we previously reported for third carpal bone cartilage PG Sepharose CL-2B profiles from animals with untreated experimental synovitis. In that study, hydrodynamically small PGs were preferentially found. Thus, the results of the present study suggested HA-induced synthesis of hydrodynamically larger PGs ($K_{av} \leq 0.30$) in animals with synovitis, which was also the pattern seen in saline-treated control horses.

The elution profiles on Sepharose CL-2B prompted an evaluation of PGs by CAPAGE. Although the distribution of peak II changed according to whether synovitis was initially present or not, as well as the time of evaluation, the reduced and alkylated equine PG monomer migrated only somewhat faster than human cartilage PG monomer and retained the HA-binding region epitope reactive with Mab 1C6. This result was independent of whether synovitis was present or not. This suggested that no overt structural alterations occurred in the equine PG monomer from horses that had synovitis and had also received HA. In addition, the faster migration position of equine peak II PG compared with fraction A1D1 on the gel was entirely consistent with its relatively larger $K_{av}$ on Sepharose CL-2B.

Mab 1C6 reacts with the HA-binding region of PGs that form aggregates with HA in the presence of link protein. Although retention of the Mab 1C6 epitope does not mitigate against the possibility that PG cleavage in equine synovitis occurred in the PG interglobular region or PG glycosaminoglycan-rich domains, the Western blot analysis suggested that HA prevented the loss of Mab 1C6 reactivity, as a previous study in untreated equine synovitis resulted in a PG from peak II that did not react with Mab 1C6. This novel finding of the present study is especially pertinent because surgical synovectomy following induction of acute synovitis had an effect on PG structure similar to that of HA, in that there was an increase in the relative proportion of larger PGs on Sepharose CL-2B compared with non-synovectomized animals. The PG from synovectomized animals was also reactive with Mab 1C6.

Indentation testing provides a powerful tool for evaluating the extent to which alterations in the biomechanical properties of articular cartilage result from exercise, inflammation or other microenvironmental stresses to the joint. The measured parameters, aggregate modulus, Poisson’s ratio, permeability and cartilage thickness can also be useful in determining the extent to which extracellular matrix contributes to cartilage responses to changes in the joint microenvironment. We have previously addressed the interpretation of how these measurements contribute to an understanding of cartilage biomechanical properties.

Previously we showed that, among the four parameters of cartilage biomechanical properties measured by indentation testing of third carpal bone cartilage, Poisson’s ratio was lower in untreated, experimentally induced equine synovitis compared with cartilage from sham saline-treated control animals. Since cartilage PGs provide the physiochemical mechanism for cartilage resistance to compression, a low Poisson's ratio is an indicator of greater cartilage compressibility, presumably as a result of changes in PGs. By contrast, the cartilage was thicker from animals with untreated synovitis compared with control animals, which we attributed to location-dependent swelling in response to changes in extracellular matrix composition as suggested by Pritzker. In the present study, Poisson's ratio, aggregate modulus, permeability and tissue thickness did not differ among the groups. Despite the fact that synovitis reduced PG content in HA-treated horses at the 2-week evaluation, the present findings suggested that HA sufficiently protected cartilage extracellular matrix as to negate the effect of reduced PG content on tissue compressibility and thickness. Taken together with the results of the PG analysis, the present results indicated that while HA did not provide a short-term benefit to cartilage PG metabolism in this model of synovitis, HA did cause a rebound effect to occur, as by 6 weeks depressed cartilage metabolism parameters returned to similar levels as in joints without synovitis.

Finally, additional studies employing HA of differing molecular weights are warranted to determine the extent to which early third carpal bone cartilage responses to mid-range molecular weight HA (0.5 x 10$^6$) alone, or in the context of synovitis, may improve when high-molecular-weight HA is employed. Indeed, a previous study suggested that intra-articular HA of high molecular weight (1.9 x 10$^6$) was more effective than HA of lower molecular weight (0.8 x 10$^6$) (which is similar in size to the Hylartin-Y preparation used here) in inhibiting femoral condyle and tibial plateau degeneration in a medial meniscectomy model of osteoarthritis in the rabbit.

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