Effects of Intraarticular Hyaluronan on Matrix Changes Induced in the Lateral Meniscus by Total Medial Meniscectomy and Exercise

By Nigel Hope, Peter Ghosh, Thomas K.F. Taylor, Dechang Sun, and Richard Read

Total medial meniscectomy was performed in 12 adult merino sheep. Immediately after surgery, 8 animals received high-molecular-weight hyaluronan (HA) (1 mL, 10 mg/mL) and 4 were given sterile saline (1 mL) intraarticularly. Injections were given for 5 more weeks. In week 3 an exercise program, consisting of walking 24 km/wk, was initiated. This program was continued until the animals were killed at week 26 postmeniscectomy. At necropsy the lateral menisci were removed and divided into three concentric zones—inner, middle, and outer. Powdered aliquots of tissues from each zone were analyzed for collagen and hexuronate contents using colormetric methods. The glycosaminoglycans (GAGs)—chondroitin-0-sulfate (C-0-S), chondroitin-4-sulfate (C-4-S), chondroitin-6-sulfate (C-6-S), and dermatan sulfate (DS)—were determined using a high-performance liquid chromatography method. The lateral menisci from the joints of animals injected with HA showed higher hexuronate and GAG levels than those of controls. This increase was mainly due to C-6-S, which had highest levels in the inner and middle meniscal zones. In addition, dermatan sulfate levels increased significantly in the middle and outer zones of the lateral menisci compared with the same zones of the meniscus from the saline-treated group. Collagen and C-O-S levels were not statistically different from those of controls. These data suggest that intraarticular administration of high-molecular-weight HA immediately after open total medial menisectomy may help preserve the proteoglycans in the lateral meniscus remaining in the joint.

INDEX WORDS: Meniscectomy; meniscus; proteoglycans; exercise; hyaluronan.

THE MENISCI (semilunar cartilages) are important structures for optimal function of the knee joint. They are shock-absorbing tissues which transmit up to 60% of the loads acting across the joint.[1-5] Apart from reducing the contact stresses on articular cartilage by increasing congruency between the curved femoral and tibial surfaces, they stabilize the joint both laterally and anterior-posteriorly.[6,7] In flexion and extension the menisci are displaced anterior-posteriorly[7] and their movement during extension and flexion is believed to drag synovial fluid over the articular cartilage surface, thereby facilitating its nutrition and lubrication.[6,8]

A meniscus may be injured or torn, particularly during sporting activities; if the damaged structure interferes with normal joint function and produces pain, meniscectomy may be indicated. However, it is now recognized that meniscectomy is not a benign procedure. Total, and to a lesser extent partial, excision of a meniscus would, on the grounds discussed above, increase the contact and shear stresses acting on articular cartilage, which could lead to cartilage degeneration. Indeed, meniscectomy in rabbits,[9-12] dogs,[13-16] and sheep[17-20] has been used for many years to produce experimental models of osteoarthritis (OA). Furthermore, retrospective clinical studies[21-24] have demon-

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strated a high incidence of OA in humans after meniscectomy.

Although the degenerative changes that occur in the matrix of articular cartilage as a consequence of meniscectomy have represented a focus of research, data on the effects of unicompartmental meniscectomy on the composition and structure of the remaining meniscus could also be important. Such knowledge could contribute to a better understanding of the remodeling response of fibrocartilage to altered mechanical loading. In addition, if the composition of the remaining meniscus is altered as a consequence of meniscectomy its ability to absorb and dissipate mechanical stresses could be modified.[1-4]

Intraarticular hyaluronan (HA) has been used for more than 20 years for viscosupplementation of synovial fluid in posttraumatic equine OA and idiopathic OA in humans with reported clinical benefits.[25] More recently, intraarticular HA has been used in arthroscopic surgery to reduce adhesions and scar formation.[26-28] Although the effects of intraarticular administration of high-molecular-weight HA on the articular cartilage of medially meniscectomized joints have been investigated,[19,20] the influence of such treatment on the composition of the remaining lateral meniscus has not been examined. The present study was undertaken to address this question using an established meniscectomy model in sheep.[17-20]

MATERIALS AND METHODS

Twelve purebred adult Merino wethers were studied. All animals underwent unilateral medial meniscectomy using a procedure described previously.[17] Before meniscectomy, 8 of the 12 sheep were randomly selected to receive HA. Immediately after surgery, when the surgical opening had been sutured, each animal received intraarticularly 1.0 mL (10 mg/mL) of sterile high-molecular-weight HA (Healon; molecular weight, $\sim 3.5 \times 10^6$) preparation, which was kindly provided by Dr G. Smedegård of Pharmacia AB, Uppsala, Sweden. The remaining four meniscectomized sheep were each given 1.0 mL of sterile saline by the same route as for the HA injections.

For the next 2 weeks all animals were maintained in a communal pen (3 m × 6 m), and each received a single weekly intraarticular injection of HA or saline. Three weeks postmeniscectomy, when all animals had received three injections, they began an exercise regime. This consisted of walking the group (3 to 4 km/h) around a level 2-km oval track covered in loose gravel and grass. Over the following 2 weeks the number of circuits was increased until the animals walked 8 km/d, three times a week. This exercise program of 24 km/wk was maintained for the duration of the experiment, ie, 6 months postmeniscectomy. During the first 3 weeks of track work each animal received a single weekly injection of either HA or saline. Therefore, each animal received a total of six injections postmeniscectomy.

Dissection and Tissue Preparation

Animals were killed by intravenous infusion of sodium pentobarbitone (60 mg/mL) (Nembutal; Abbott Laboratories, Sydney, Australia). Lateral menisci were dissected from the meniscectomized joints, rinsed in ice-cold physiological saline, dried with a paper tissue, transferred to airtight plastic bags, and frozen until required. Before analysis each meniscus was thawed at 4°C and dissected into three longitudinal sections corresponding to the inner, middle, and outer zone of the meniscus. Each zone represents a concentric one third of the meniscus, as described previously.[29] Each zone was then refrozen in liquid N_2 and powdered in this medium using a Wiley Mill (A.J. Thomas, Philadelphia, PA) in which the cutting chamber had been precooled with liquid N_2. The individually powdered meniscal zones were transferred to glass containers, lyophilized, stoppered, and stored in desiccated containers at 4°C until analysis.

Analysis

Preweighed aliquots of lyophilized powdered meniscal tissues were hydrolyzed in 6N HCl by heating at 100°C for 16 hours. The hydroxyproline released was determined using the method of Stegeman and Stalder.[30] The collagen content was estimated from these values by multiplying by 7.4.[29] Aliquots were also subjected to papain digestion[31] and the hexuronic acid levels determined by the method of Blumenkrantz and Asboe-Hansen[32] using glucuronyl lactone (Sigma Chemical Co, St Louis, MO) as a standard. The glycosaminoglycans (GAGs) present were identified and quantitated according to the high-

performance liquid chromatography (HPLC) method described below.

Sample Preparation for GAG Analysis

The method used to identify and quantitate cartilage GAGs was based on that described by Greiling et al.[33] However, modifications were made to minimize the number of sample manipulations and facilitate analysis of large numbers of samples. Aliquots (~5 mg) of lyophilized tissue were weighed into 1.5-mL Eppendorf tubes. Two hundred fifty microliters of papain digestion buffer containing 0.1 mol/L Na-acetate, 0.005 mol/L ethylenediaminetetraacetic acid (EDTA), 0.01 mol/L cysteine, and 2 μL/mL papain suspension (Sigma Chemical Co) was added, and the tissues were hydrolyzed overnight at 60°C. One milliliter of absolute ethanol was added to the papain digest, and the GAGs precipitated overnight at 4°C on an orbital shaker at 150 rpm. The precipitate was collected by centrifugation and the supernatant decanted. The precipitated GAGs were vacuum-dried before being redissolved in 400 μL of chondroitinase digestion buffer consisting of 0.3 mol/L Na-acetate, pH 6.5. Aliquots (180 μL each) of enzyme digest were pipetted into 0.7-mL tapered glass crimp-top vials (Chromacol Ltd, London, England) which were compatible with a LS-3200 LC Auto-Sampler (SGE Scientific Pty Ltd, Melbourne, Australia). Ten microliters of 0.2 mol/L NaF and 10 μL (0.05 U) each of chondroitin sulfate lyase AC or ABC (Sigma Chemical Co) were added, and the tubes were crimp sealed and incubated overnight at 37°C.

HPLC

The unsaturated disaccharides derived from the GAGs as the end product of depolymerization with either chondroitin sulfate lyase AC or ABC were fractionated by HPLC and spectrophotometrically detected at 232 nm using a semiautomated HPLC system. This consisted of a Dynamax NH2-column (4.6 × 250 mm) fitted with a guard column (4.6 × 15 mm) (Rainin Instrument Co Inc, Woburn, MA) that was eluted with an aqueous buffer consisting of 0.3 mol/L ammonium acetate and 20 mmol/L glycine, pH 5.5, at a flow rate of 1 mL/min provided by a ETP/Kortec K25M single-piston pump (ICI Instruments, Rydalmere, NSW, Australia). The ultraviolet detector was an ERMA model ERC-7210 (ERMA Optical Works Ltd, Tokyo, Japan), and signal analysis and peak integration were performed using a SMAD/SMAD chromV.2.13 system (Morgan Kennedy Research, Rose Bay, NSW, Australia) controlled by an Apple Macintosh computer.

Pure standards of HA (Healon; Pharmacia [Australia] Pty Ltd, North Ryde, NSW, Australia), chondroitin-4-sulfate (C-4-S) (Calbiochem, Alexandria, NSW, Australia), and disc chondroitin-6-sulfate (C-6-S) donated by Professor Pearce, University of British Columbia, Vancouver, Canada, and dermatan sulfate (DS) (Sigma Chemical Co) were used for the quantitation of GAGs. The amount of DS present in each GAG precipitate was calculated by the difference of 4-sulfated disaccharide values obtained by chondroitin sulfate lyase ABC and AC digestion.

Methods of Statistical Analysis

Because four control and eight HA-injected animals were used for this study, analytical data were analyzed using the unpaired two-tailed *t* test. Differences between HA- and saline-treated groups were considered statistically significant at $P \leq .05$.

RESULTS

At necropsy a partially regrown meniscus replica was found in the medial compartment of all animals. This structure was integrated with the synovium from which it probably originated.[15] No significant differences were observed between the experimental groups in terms of the size or mass of these regrown tissues (data not shown). As noted previously,[29] the outer region of the menisci contained more collagen than the inner, but differences were not observed between the levels of this protein in the same zones of menisci from placebo (saline)- or HA-injected joints (data not shown). In contrast, the hexuronate values of the inner and middle zones of the lateral menisci derived from HA-injected joints were 50% to 100% higher than those from the same zones of menisci from the placebo-injected animals ($P < .05$) (Fig 1). This elevation in hexuronate values was also mirrored in total GAG levels as deter-

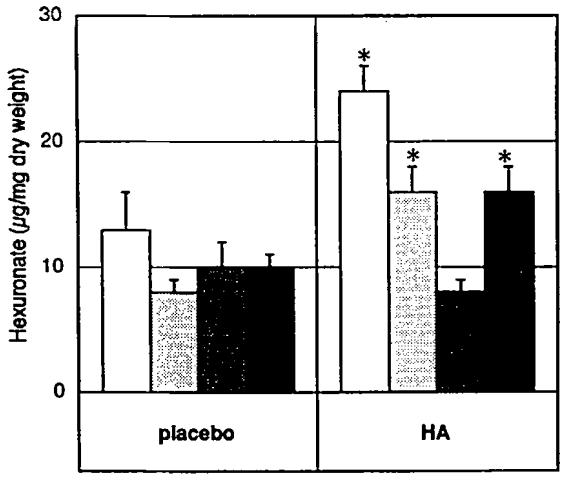

Fig 1: Hexuronate content (μg/mg dry weight of tissue) of the various zones of lateral menisci from meniscectomized joints of animals injected intra-articularly with saline (placebo) or HA (mean ± SE). Meniscal zones were defined as inner (□), middle (▨), outer (▩), and pooled (■). Statistically significant differences (*$P < .05$) existed between the groups for the inner, middle, and pooled zones.

mined by HPLC, but for these analyses statistical significance ($P < .05$) was demonstrable between the groups only for data pooled from all meniscus regions (Fig 2).

The comparison of individual GAGs of the menisci of the two groups showed no difference in their chondroitin-0-sulfate (present mainly as HA) content (Fig 3), but marked differences in the other chondroitin sulfate isomers were observed. C-4-S levels were elevated in the outer meniscal zone of the HA-injected group compared with the same zone of the placebo-treated group ($P < .05$) (Fig 4). C-6-S levels were higher in the same tissues as C-4-S, but again in the middle region of the menisci from the HA-injected animals (Fig 5). DS, although less abundant than the other chondroitin sulfate isomers, was elevated in the middle and outer zones of the lateral menisci of the HA-injected joints compared with the same zones of the menisci in the placebo group ($P < .05$) (Fig 6).

DISCUSSION

The knee joint menisci are fibrocartilaginous tissues whose composition and structure are well adapted for their functions. Because they are wedge-shaped in cross-section and the coefficient of friction of synovial fluid is low, they are squeezed outwardly when the joint is loaded. Radial displacement of the menisci is resisted by their tibial attachments, and a circumferential "hoop strain" develops in the periphery of the structure on joint loading.[4,34] To accommodate these high tensile stresses, the outer rim of the meniscus is composed predominantly of thick collagen fibers aligned circumferentially. The inner and middle zones of the meniscus, while still rich in collagen, contain much higher amounts of proteoglycans than the outer zone.[15,29,35] The composite structure of the meniscus is thus well suited to withstand and recover from both compressional and tensional stresses.[15,36] Biochemically and morphologically,[29,35,37] the inner and middle zones of the meniscus show many similarities to hyaline articular cartilage except that the proteoglycan content is lower and the collagen present is largely type I rather than type II.[35]

Studies by Tribe[38] and Squire et al[39] on grazing patterns of sheep have shown that on average these animals traverse 4 km/d in fenced paddocks

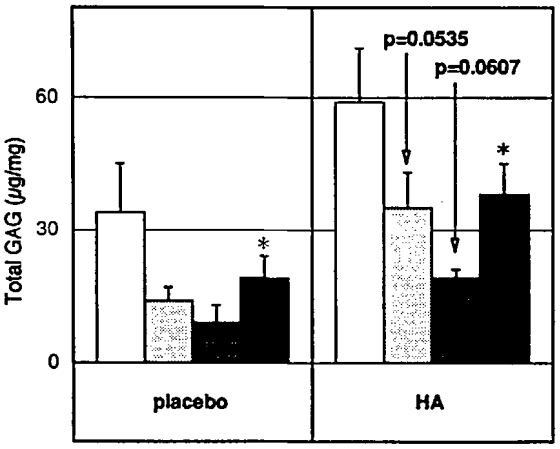

Fig 2: Total GAG content (μg/mg dry weight of tissue) of various zones of lateral menisci from meniscectomized joints of animals injected intra-articularly with saline (placebo) or HA (mean ± SE). Meniscal zones were defined as inner (□), middle (▨), outer (▩), and pooled (■). Statistically significant differences (*$P < .05$) existed between the groups for the pooled zones.

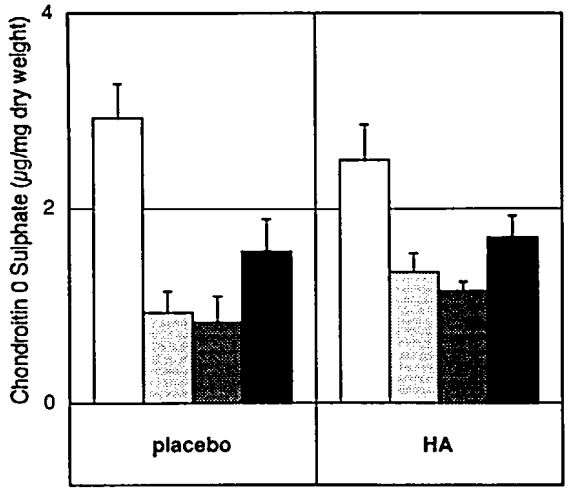

Fig 3: Chondroitin-O-sulfate (mainly HA) content (μg/mg dry weight of tissue) of lateral menisci from meniscectomized joints of animals injected intra-articularly with saline (placebo) or HA (mean ± SE). Meniscal zones were defined as inner (□), middle (▨), outer (▩), and pooled (■).

irrespective of the area. The exercise regimen used in the present experiments, in which sheep were walked 24 km/wk around a grass track, is therefore comparable with their normal activity pattern, bearing in mind that some movements also occurred while the animals were penned.

In a previous study[40] it was shown that the hexuronate contents of all zones of both the medial and lateral menisci of unoperated sheep subjected to an exercise program similar to that used in the present experiments were elevated compared with the same zones of a nonexercised, nonoperated group. This finding shows that the fibrochondrocytes of the knee joint menisci, like the chondrocytes of articular cartilage,[41,42] can adapt to a new mechanical environment by modifying biosynthetic activities to elaborate an extracellular matrix more suited to the types of stresses to which they are subjected.

The most notable difference between the outcomes for the two postoperative regimens used in the present experiments was the higher concentration of proteoglycans in all zones of the lateral menisci of the HA-injected group (Figs 1 and 2). This increase could be interpreted, on the basis of previous studies,[40-42] to indicate that in the HA-treated group the lateral menisci were subjected to higher dynamic compressional stresses than the corresponding menisci of the saline-treated group. The finding that C-6-S was the major contributor to the elevated hexuronate values, particularly in the middle zone (Fig 5), was compatible with this reasoning.[41,42] Increased loading of the lateral menisci after 6 weekly injections of high-molecular-weight HA could arise from improved weight bearing on the meniscectomized joint because force-plate studies using the same ovine model used here, but with HA preparations of lower molecular weight, showed that lameness was diminished in the actively treated groups compared with saline controls.[18,19]

On the other hand, the immediate postoperative injection of HA into the meniscectomized joints may have preserved meniscal proteoglycan levels directly. High-molecular-weight HA is reported to encourage healing of experimentally created defects in rabbit anterior cruciate ligaments.[43] However, the rate of healing of circular defects in rabbit menisci after a single injection of HA into the joints was found to be indistin-

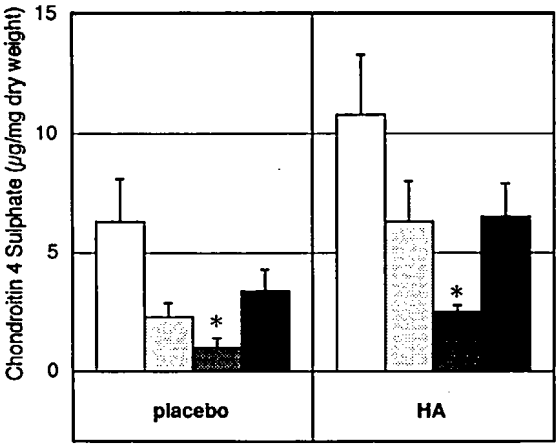

Fig 4: C-4-S content (μg/mg dry weight of tissue) of the various zones of lateral menisci from meniscectomized joints of animals injected intraarticularly with saline (placebo) or HA (mean ± SE). Meniscal zones were defined as inner (□), middle (▨), outer (▩), and pooled (■). Statistically significant differences (*$P < .05$) existed between the outer zones of the two groups.

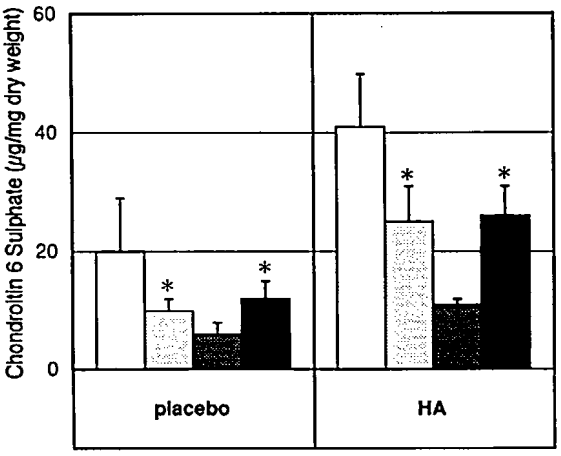

Fig 5: C-6-S content (μg/mg dry weight of tissue) of the various zones of lateral menisci from meniscectomized joints of animals injected intraarticularly with saline (placebo) or HA (mean ± SE). Meniscal zones were defined as inner (□), middle (▨), outer (▩), and pooled (■). Statistically significant differences (*$P < .05$) existed between groups for the middle and pooled zones.

guishable from that of saline controls.[44] The reduced proteoglycan contents of the lateral menisci removed from saline treated joints could have initially arisen as a consequence of enhanced matrix catabolism. This may have resulted from the postoperative trauma and local inflammation associated with open meniscectomy. In this regard the documented antiinflammatory properties of HA,[45-48] coupled with its ability to attenuate oxygen-derived free radical[49] and proteolytic[49-52] breakdown of cartilage may have provided some protection to meniscal proteoglycans.

The C-6-S and C-4-S of human[35,37] and canine[35] menisci have been analyzed and would appear to be present in these tissues as large aggregating proteoglycans (aggrecans) similar to those isolated from hyaline cartilage.[35,37] Indeed, they may be encoded from the same gene.[37] Similarly, the elevated levels of DS in the middle and outer zones of the menisci of the HA-treated group presumably arise from an increase in DS-containing proteoglycans (DS-PGs). Two DS-PGs, decorin and biglycan, have been isolated from human menisci.[37] Biglycan consists of a core protein of molecular weight ~42,500 to which two DS chains (molecular weight ~37,000) are attached. Decorin has a different core protein to biglycan and has only one DS chain. The DS chains for both DS-PGs are hybrids containing iduronic and glucuronic acid residues glycosidically linked mainly to 4-sulfated *N*-acetyl galactosamine (see reviews[53,54] for collected references).

Although the biological role of the DS-PGs is still the subject of research, it is clear that they have many functions. It has been shown that decorin is associated with the d band of type I and II collagen fibrils and can inhibit fibrillogenesis in vitro.[54] Cartilage-derived decorin and biglycan have been shown to inhibit attachment and spreading of 3T3 cells to a fibronectin-coated matrix.[53,55] However, because neither the DS chains nor the core proteins alone could inhibit cell attachment, it was concluded that the DS-PG complex was necessary for inhibition. These studies have led to the proposal that the DS-PGs bind to a specific receptor on fibronectin adjacent to the integrin-binding site. When DS-PGs are

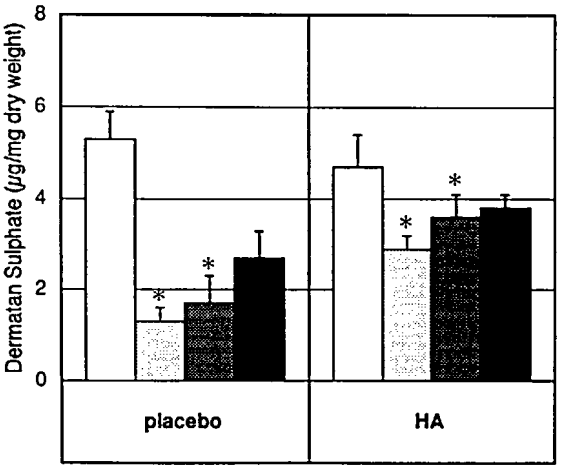

Fig 6: DS content (μg/mg dry weight of tissue) of the various zones of lateral menisci from meniscectomized joints of animals injected intraarticularly with saline (placebo) or HA (mean ± SE). Meniscal zones were defined as inner (□), middle (▨), outer (▩), and pooled (■). Statistically significant differences (*$P < .05$) existed between groups for the middle and outer zones.

bound to this receptor, the DS side chains sterically block the integrin-binding receptor, thereby inhibiting cell adhesion. Additional interactions between the GAG chains and fibronectin may augment this process.[53] Decorin can also bind transforming growth factor β (TGF-β) via its coreprotein[56] and modulate the activities of this ubiquitous growth factor. This has led to the suggestion that decorin may be a regulator of cell proliferation. However, TGF-β also stimulated the synthesis of biglycan and decorin by fibroblast and ovary cells,[56,57] and because DS-PGs can bind TGF-β a negative-feedback regulation of cell mitosis has been proposed.[56] These and other[58] experiments have led to the suggestion that the DS-PGs inhibit connective tissue repair processes.[53]

Dynamic loading (5 kg/0.3 Hz) of bovine sesamoid cartilage in vitro stimulates the biosynthesis of DS-PGs, especially decorin.[59] Similar results have been obtained for degenerative articular cartilage from adult sheep joints subjected to lateral meniscectomy.[60] The deposition of DS-PGs in hyaline cartilage therefore would seem to be associated with chondrocyte response to high dynamic loading and presumably represents part of an adaptive reorganization of the extracellular matrix in response to mechanical overload. Whether the enrichment of the lateral menisci of the HA-treated joints with DS-PGs is also related to altered mechanical loading of this tissue or arises from a direct pharmacological effect of HA has yet to be determined. However, the short half-life of HA in synovial joints[61,62] suggests that either or both of these mechanisms would provide the maximum benefit during the immediate postoperative period, when tissue remodeling and repair are most active.

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