**Significant synovial pathology in a meniscectomy model of osteoarthritis: modification by intra-articular hyaluronan therapy**

M. M. Smith¹, M. A. Cake², P. Ghosh¹, A. Schiavinato³, R. A. Read² and C. B. Little¹

**Objective.** IA therapy with hyaluronan (HA) is reported to provide symptomatic relief and disease modification in OA. This study assessed the pathological changes in the synovium of an ovine model of OA and evaluated the effects of two HA preparations on this pathology.

**Methods.** Eighteen sheep had bilateral lateral meniscectomy to induce OA. Four months post-surgery animals received IA saline or HA (Hyalgan®) weekly for 5 weeks or three injections of an amide derivative of HA (HYADD®-4-G) every 2 weeks (n=6 per group). Six months after meniscectomy, sheep were killed, knee joint synovium processed, scored for pathological change and compared with synovium from non-operated animals. Sections of synovium from normal and treated joints were also immunostained for TNF-α, HSP-47, TGF-β, CD44, connective tissue growth factor (CTGF) or iNOS. HA synthesis by synovial fibroblasts isolated from each OA joint was quantified.

**Results.** Aggregate scores of pathological change were higher in OA joint synovia compared with controls, with individual measures of subintimal fibrosis and vascularity predominantly affected. Depth of intimal fibrosis was also significantly higher in meniscectomized joints. IA treatment with Hyalgan® decreased aggregate score, vascularity and depth of fibrosis. HYADD®-4-G treatment decreased vascularity, intimal hyperplasia and increased high-molecular weight HA synthesis by synovial fibroblasts. CD44, CTGF or iNOS expression was increased in the synovial lining of OA joints compared with normal, but there was no significant modulation of this increase by either HA preparation.

**Conclusion.** Increased fibrosis and vascularity are hallmarks of pathological change in synovium in this meniscectomy model of OA. Both the IA HA and an amide derivative of HA reduced aspects of this pathology thus providing a potential mechanism for improving joint mobility and function in OA.

**Key Words:** Osteoarthritis, Animal model, Synovium, Hyaluronan.

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its relatively short half-life within the joint cavity (<24 h) [25]; therefore, modified HA preparations, such as the amide derivative HYADD\(^4\)-G, are being manufactured, which have a longer joint residency but retained SF-like viscoelastic properties [26].

To date there is little data on the effect of HA on synovial pathological changes with OA in animal models. We have used a well-established OA model induced by meniscectomy in sheep to test efficacy of many potential therapies [27], including several trials of IA HA preparations which have variously been shown to reduce cartilage histopathology scores [28, 29], improve gait [30] and increase osteophytosis [31, 32]. However, synovial pathology has not routinely been assessed in these studies.

We have developed and validated a histological scoring system for synovium from OA joints to quantitate pathological changes in this animal model [33]. In the present study, we confirm that there are significant changes to ovine synovium after meniscectomy and have investigated potential mechanisms for the resulting pathology. Furthermore, we evaluate the relative effects of IA Hyalgan\(^4\) and HYADD\(^4\)-G, an amide derivate of Hyalgan\(^8\), on the histopathology, immunohistochemistry and ex vivo endogenous HA synthesis of synovium derived from stifle joints of meniscectomized sheep.

### Methods

#### Animal experimentation

Eighteen aged (7- to 8-ys old) Merino ewes were subjected to bilateral lateral meniscectomy as previously described [34, 35]. Two HA preparations were tested vs saline placebo, Hyalgan\(^8\) (10 mg/ml) and HYADD\(^4\)-G (5 mg/ml), both manufactured and supplied by Fidia Farmaceutici S.p.A., Abano Terme, Italy. Sheep were randomly allocated prior to surgery to one of the three treatment groups (n = 6 per group): OA + saline placebo, OA + Hyalgan\(^8\) and OA + HYADD\(^4\)-G. From weeks 16 to 20 post-meniscectomy, these groups respectively received bilateral IA injections of equivalent volume (2 ml) of sterile normal saline (weekly), Hyalgan\(^8\) (weekly) or HYADD\(^4\)-G (every 2 weeks, at weeks 16, 18 and 20 post-surgery). IA injections were performed under short-acting deep sedation (intravenous diazepam at 0.25 mg/kg with ketamine 5 mg/kg) using a 21-gauge needle and aseptic conditions. Post-operatively, animals were transferred to irrigated pasture (1 hectare paddocks) on the Murdoch University farm, partially supplemented with lucerne chaff and lupins in order to maintain constant body condition. Sheep were monitored daily and killed at 6 months post-meniscectomy. All animal procedures were approved by the Murdoch University Animal Ethics Committee (AEC R779/00).

#### Tissue collection

After opening the knee joint by severing the cruciate and collateral ligaments, a sample of synovium from the suprapatellar fold was removed and placed in 10% (v/v) neutral buffered formalin. Further samples of synovia were removed from the same area and from adjacent to the femoral condyles, taking care to minimize the amount of fibrous synovium and subsynovium. These samples were processed for cell isolation as described subsequently.

#### Histological processing of synovium

Synovial specimens were fixed in 10% (v/v) neutral-buffered formalin for 24 h. Samples were then transferred to 70% (v/v) ethanol and routinely processed through ascending grades of ethanol (70–100% (v/v)), cleared in chloroform, then infiltrated and embedded in paraffin wax. Sections (4 μm) were cut on a rotary microtome and stained with haematoxylin and eosin (H&E).

### Immunohistology

Immunostaining for CD44, TGF-\(\beta\), CTGF, TNF-\(\alpha\), inducible NO synthase (iNOS) and HSP-47 was performed on sections from one joint of each sheep as well as age-matched identically processed NOC synovial specimens. Endogenous peroxidase activity was initially blocked by incubating the tissue sections with 3% (v/v) H\(_2\)O\(_2\) for 5 min.

Some sections (for TGF-\(\beta\), TNF-\(\alpha\) and iNOS immunostaining) were pre-digested with bovine testicular bovine hyaluronidase (600 U/ml) for 30 min at 37°C in phosphate buffer, pH 7.4; for CD44 immunostaining, heat retrieval was performed in 0.01 M citrate buffer, pH 6.0 for 20 min at 99°C (water bath) followed by 10 min cooling at 22°C. Non-specific binding was blocked by incubating the sections in non-serum protein block (DakoCyto-mation, USA) for 10 min at room temperature. Incubations were performed overnight at 4°C with primary antibodies for CD44 (rat monoclonal; Serotec MCA1449; 1:20 dilution), CTGF (rabbit polyclonal; Abcam ab6992; 1:1000 dilution), iNOS (rabbit polyclonal; Cayman Chemicals 160 862; 1:1000 dilution) and TNF-\(\alpha\) (rabbit polyclonal; Chemicon AB1842; 4-G, an amide derivate of Hyalgan\(^4\)-G (5 mg/ml), both manufactured and supplied by Fidia Farmaceutici S.p.A., Abano Terme, Italy. Sheep were randomly allocated prior to surgery to one of the three treatment groups (n = 6 per group): OA + saline placebo, OA + Hyalgan\(^8\) and OA + HYADD\(^4\)-G. From weeks 16 to 20 post-surgery, IA injections were performed under short-acting deep sedation (intravenous diazepam at 0.25 mg/kg with ketamine 5 mg/kg) using a 21-gauge needle and aseptic conditions. Post-operatively, animals were transferred to irrigated pasture (1 hectare paddocks) on the Murdoch University farm, partially supplemented with lucerne chaff and lupins in order to maintain constant body condition. Sheep were monitored daily and killed at 6 months post-meniscectomy. All animal procedures were approved by the Murdoch University Animal Ethics Committee (AEC R779/00).

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HA synthesis as described subsequently. Fields per section were calculated. At 37 °C for 3 h and then at 94 °C for 16 h. Changes in the amount of 3H-glucosamine incorporated into endogenous HA were measured as radioactivity peaks separated by scintillation spectrometry in Ecolite® (ICN Biochemicals). CPM in hyaluronidase-digested samples was subtracted from CPM in undigested samples to obtain CPM specifically due to 3H-hyaluronan. The CPM value thus obtained for each well was corrected for number of cells present, as determined by the cellular DNA content. Total HA synthesised by the cells was determined from all Superose 6 fractions before the total column volume in SHase-digested fractions whereas HMW-HA was determined from CPM that voided the column.

**Statistical methods**

Non-parametric data (histological and immunohistological scoring) was analysed using the Kruskal–Wallis test for multiple groups and, if significance was found, Mann Whitney U-tests for between-group comparisons (n=12 joints per treatment) were used.

The α-level was set at 0.05, reducing to 0.03 following Benjamini–Hochberg post-hoc procedure for multiple comparisons [38] to correct for Type 1 errors. For parametric data (intimal fibrosis and HA synthesis), unpaired Student’s t-tests were performed, with P<0.05 considered to be statistically significant.

**Results**

**Effect of meniscectomy—histological scoring**

Marked differences were observed between knee joint synovia from NOC animals and saline-treated meniscectomized animals. Subintimal fibrosis (P=0.0035), vascularity (P=0.0035) and aggregate score of the non-parametric criteria (P=0.0036), were all significantly higher in OA joint synovia (Table 2). The increase in intimal hyperplasia was not statistically significant (P=0.044) while the increase in cellular infiltrate was highly variable (P=0.13). Depth of intimal fibrosis (P=0.010) was significantly increased in synovium from meniscectomized saline-injected joints compared with NOC (Fig. 1).

**Effect of meniscectomy—immunohistology**

In the synovium of NOC joints, intimal cells and occasional fibroblastic subsynovial cells were positive for HSP-47. In contrast, TGF-β and TNF-α were localized to blood vessels as well as occasional intimal cells. Immunostaining for HSP-47 was unaltered in any meniscectomized joints and although a slight increase in intimal cell staining for TGF-β and TNF-α was observed in some OA joints, this was highly variable and was not significant when sections were blindly scored (data not shown).

In NOC sheep, the antibody for CD44 stained the superficial synovial intima only, with staining deeper into the tissue rarely seen (Fig. 2D). Occasional lining cells had cell membrane staining.

### Table 2. Histological scores for H&E sections of stifle joint synovium sampled from NOC sheep and OA sheep subjected to various IA treatments (scored as described in Table 1)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intimal hyperplasia</th>
<th>Cellular infiltrate</th>
<th>Subintimal fibrosis</th>
<th>Vascularity</th>
<th>Aggregate scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOC</td>
<td>2.83 ± 0.31</td>
<td>0.83 ± 0.40</td>
<td>3.33 ± 0.33</td>
<td>3.50 ± 0.43</td>
<td>7.50 ± 0.71</td>
</tr>
<tr>
<td>OA + saline</td>
<td>4.00 ± 0.37</td>
<td>3.33 ± 1.09</td>
<td>3.67 ± 0.49</td>
<td>5.83 ± 0.17</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>P (vs NOC)</td>
<td>0.044</td>
<td>0.13</td>
<td>0.0035</td>
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</tr>
<tr>
<td>OA + HYADD4-G</td>
<td>3.00 ± 0.52</td>
<td>1.67 ± 0.61</td>
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<td>P (vs NOC)</td>
<td>1.00</td>
<td>0.27</td>
<td>0.0049</td>
<td>0.14</td>
<td>0.0046</td>
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<tr>
<td>P (vs OA + saline)</td>
<td>0.14</td>
<td>0.19</td>
<td>0.32</td>
<td>0.020</td>
<td>0.015</td>
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<tr>
<td>OA + HYADD4-G</td>
<td>2.33 ± 0.56</td>
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<td>3.33 ± 0.80</td>
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Values are mean ± s.e.m, n=12 joints. P-values by Mann–Whitney U-ranked tests, with P<0.03 statistically significant after Benjamini–Hochberg post-hoc correction. There were no significant differences between Hyaligan®- and HYADD®-G-treated groups (P>0.5).

### Table 1

| Treatment     | HA synthesis by synovial fibroblasts | 3H-glucosamine (specific activity = 8.80 Ci/mmol; GE Healthcare) | was added to DMEM/10% FBS at 1 µCi/ml. Two millilitres were added to each well (2 µCi per well) and exactly 24 h later, media was removed from cells, the cells rinsed with PBS and harvested for DNA determination using a modification of the Hoescht 33258 dye-binding method described by Kim et al. [36] and Cakc et al. [37]. After adjusting the pH to 6.0 with 10 µl 1 M acetic acid, 50 µl 20 mM sodium acetate/0.15 M NaCl pH 6.0 with and without 5 TRU Streptomyces hyaluronidase (SHase) was added to 500 µl aliquots of the OSF suspension per joint; were placed into 3 x 10 cm² wells of 6-well plates and incubated at 37 °C. Media was changed every 2 or 3 days. When the cells were ~70–80% confluent, they were used to determine de novo HA synthesis as described subsequently.**

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but, as most lining cells are very elongated and fibroblastic, this was not always clear. In OA joints, the immunostaining was cell membrane-associated in all cases and appeared to be strongest on the cell membranes adjacent to the synovial space, with little staining in the subsynovial tissue. Synovial lining cells and some cells in the subsynovium were positive for CTGF in NOC joints (Fig. 2E). Staining appeared to be mainly cytoplasmic and varied between cells from no staining to very heavy staining within the same section of tissue. In OA joints, the tissue distribution of CTGF was similar to NOC but more intense staining was observed, and in addition some matrix staining was evident that was not present in the negative control sections. In synovia from NOC sheep, the antibody for iNOS stained only a very few lining cells in some specimens (not all) with no ECM or subsynovium staining in any specimen (Fig. 2F). After meniscectomy, many lining cells exhibit heavy staining, with some positive cells also appearing in the subsynovial tissue. There was more intense antibody staining for CD44, CTGF and iNOS on the synovial lining of meniscectomized joints when compared with NOC (all \( P = 0.004 \)).

**Effect of IA HA treatment**

IA treatment with either Hyalgan\(^*\) (\( P = 0.015 \), significant) or HYADD\(^*\)-4-G (\( P = 0.076 \), not significant) decreased the aggregate scores to the same extent compared with saline treatment (Table 2). Compared with saline treatment, vascularity was significantly decreased by both Hyalgan\(^*\) (\( P = 0.020 \)) or HYADD\(^*\)-4-G (\( P = 0.023 \)) while intimal hyperplasia was only reduced by HYADD\(^*\)-4-G (\( P = 0.032 \), not significant). Subintimal fibrosis score was not decreased by either HA preparation (Table 2). In contrast, intimal fibrosis depth was decreased significantly by Hyalgan\(^*\) (\( P = 0.028 \)) and not by HYADD\(^*\)-4-G (\( P = 0.078 \)) compared with saline (Fig. 1). There were no differences between IA treatments with either Hyalgan\(^*\) or HYADD\(^*\)-4-G in any of the synovial pathological changes \( (P > 0.5 \) for all variables measured). Neither of the HA treatments modulated intensity or distribution of immunostaining for any of the molecules examined (Fig. 2J–O).

**FIG. 1.** Depth of fibrosis of the synovial intima (mean ± s.d., \( n = 12 \) joints per group). There were no significant differences between IA treatments \( (P > 0.28) \) (unpaired Student’s \( t \)-test).

**FIG. 2.** Representative images of immunohistology of ovine synovium from NOC (A–F), saline-treated (G–I), Hyalgan\(^*\)-treated (J–L) or HYADD\(^*\)-4-G treated (M–O) joints stained with negative control antibodies (A–C) or antibodies to CD44 (D, G, J, M); CTGF (E, H, K, N) or iNOS (F, I, L, O) as outlined in Methods section. Bar represents 100 \( \mu m \) for all images, which were captured at \( \times 200 \) magnification.
HA synthesis by synovial fibroblasts

Preliminary experiments using OSFs established that cells from the ovine species, unlike the corresponding human and lupine synovial cells, do not retain their morphology and phenotypic expression for many passages in culture (data not shown). For this reason, the HA synthesis by the isolated synovial fibroblasts from each sheep joint was determined using primary (previously uncultured) cells. There were no differences in total or HMW-HA synthesis by the synovial cells from Hyalgan®-treated joints compared with saline treatment (Fig. 3). In contrast, IA treatment with HYADD®-G showed a trend towards increased quantity of total HA (P=0.088, not significant) and a significantly increased HMW-HA (P=0.042) synthesis by the ovine synovial cells when compared with cells from the joints of saline-treated sheep.

Discussion

We have shown that there are significant pathological changes in the synovium of sheep 6 months following meniscectomy. The histological scoring system used was an extension of that previously described [33, 39] and specifically designed to detect differences in this ovine model. The changes induced by meniscectomy include increased intimal cells, intimal and subintimal fibrosis and vascularity when compared with synovium from unoperated sheep joints, which mimics the synovial pathological changes reported in human OA synovium [11]. In contrast, there was no consistent increase in plasma/inflammatory cell infiltration, which, together with the lack of increased staining for TNF-α, indicates minimal synovial inflammation at this 6-month time point in this model. It is plausible that meniscectomy may induce an early synovial inflammation that is mostly resolved by 6 months, leaving thickened intima, subsynovial fibrosis and increased vascularity as the predominant pathological changes. This agrees with the minimal synovial changes seen in OA compared with RA in humans [40]. In order to investigate potential mechanisms for the pathological changes observed we undertook immunolocalization for a variety of factors known to be involved in these processes.

Immunohistology revealed increases in CTGF, CD44 and iNOS 6 months after meniscectomy. CTGF has been implicated as a major downstream regulator in TGF-β-dependent fibrosis, particularly of liver, lungs, heart and skin (scarring) [41] and other fibroproliferative diseases. When transfected into the synovial lining of mouse knee joints, CTGF induced a transient synovial fibrosis with ECM accumulation [42], and TGF-β has been found at sites of fibrosis in patients with OA [43]. TGF-β may be expressed early to initiate matrix accumulation and induce CTGF, which is then persistently expressed as suggested in a mouse model of fibrosis [44], which may explain the lack of change in TGF-β detected at 6 months in our model. HSP-47 is a collagen chaperone that increases during fibrosis in other tissues [45], but its lack of regulation in the current study suggests that the export of collagen from the cell is not a rate-limiting step in synovial fibrosis.

CD44, a multivariate transmembrane glycoprotein known to be the principle cell surface receptor for HA [46], is present in the synovium [47] and SF [40] of OA patients and levels correlate with the degree of inflammation but not Kellgren grade [40]. Limited evidence of inflammation was noted in our sheep model despite synovial intima staining more strongly for CD44 than normal joints. The orientation of the CD44 staining to the synovial space we report in this study has previously been seen in rat temporomandibular joint synovium, where it co-localized with fibroblastic synoviocytes (Type B) and not macrophage-like (Type A) cells [48].

The increase in iNOS may be implicated in OA pathology by increasing NO, that induces inflammation in synovium [49], apoptosis of synoviocytes [50] and degradation of AC [51]. iNOS is expressed by many mammalian cells in response to inflammatory stimuli or mediators, such as IL-1 or TNF [52]. Increased TNF-α from synovium and SFs from OA patients [53] may induce increased vascularity through its effects on VEGF [54]. However, despite a mild increase in immunostaining in some joints, there was no significant difference in staining score for TNF-α in the ovine synovium (data not shown).

HA treatment is beneficial in OA patients with decreased pain and increased range of motion [18]. There are a number of potential mechanisms whereby HA may have these clinical benefits. There is evidence that HA functions as a DMOAD, potentially slowing cartilage degradation [20]. Despite previous studies where HA treatment reduced tibial cartilage lesion size [39] and histopathology scores [28, 55], there was no evidence of modulation of AC pathology after either HA treatment in the present study (data not shown). The lack of chondroprotection in the present study may be associated with the advanced age of the sheep used (7–8 yrs compared with 2–4 yrs in previous studies) and the timing of the HA administration (16 weeks after induction of OA). Decreased total and HMW-HA in SF is a consistent finding in OA [11]. IA HA therapy both directly supplements the endogenous HA concentrations and may stimulate the fibroblasts of the synovium to produce more HA of higher quality [20, 23]. In the present study, HYADD-G stimulated the synthesis of HMW-HA ex vivo and only this HA preparation decreased intimal hyperplasia, consistent with the inhibitory effect of HMW-HA on synoviocyte proliferation in vitro [56]. Previously, we showed that acute application of non-derivatized HA in vitro increased endogenous HA synthesis by synovial fibroblasts, which was dependent on the MW of the HA preparation. It is interesting that this effect was not observed in the present study 5 weeks after HA injection of non-derivatized HA, possibly due to the presence of HA turnover/clearance mechanisms that would not be present in vitro. The enhanced ability of HYADD-G to remain in the joint cavity and hence in contact with the synovium could account for its more potent effects on the MW of endogenous HA production by synovial fibroblasts isolated 5 weeks after the last injection.

HA preparations that are of a sufficient HMW have analgesic properties when injected into both animal and human joints (reviewed in [57]). This has been confirmed both clinically and in laboratory experiments measuring neural discharges in nerves of cat [58] and rat [59] joints. The present results of reduced vascularization of OA synovium by both HA preparations are consistent with the well-recognised anti-angiogenic properties of HMW-HA [60] and could contribute to disease modification and analgesia.

Increased synovial fibrosis was experimentally measured in the synovium of patients who reported pain after cruciate ligament reconstructive surgery [61] and was uniquely correlated with knee pain in those OA patients with knee symptoms [4]. Neither of the HA preparation decreased the subintimal fibrosis score that assessed overall fibrosis in the section. However, Hyalgan® decreased intimal fibrosis depth, and this could contribute to the reduction in OA pain after HA IA treatment in patients. The reduction in fibrosis seen with the IA HA injections was not reflected in reduced CTGF levels, suggesting alternate mechanisms for the anti-fibrotic effect. CTGF has been shown to up-regulate tissue inhibitor of metalloproteinase-1 (TIMP-1) in mouse synovium [42], potentially preventing collagen catabolism by MMPs and facilitating matrix accumulation [62]. HMW-HA decreased TIMP-1 expression in isolated human synovial fibroblasts [63], and hence may allow increased MMP activity, normalizing collagen turnover and thus preventing the fibrosis. Whether the injected HA in the present joints may act to reduce TIMP-1 levels, allowing uninhibited MMP activity to counteract the effects of CTGF and thus decrease fibrosis requires further investigation.
processes by which the different HA treatments are generating modified immunostaining of the six potential effector molecules present in synovial fibroblasts isolated from knee joints of OA sheep subjected to different IA treatments. Values are mean ± s.d. (n=6 sheep, two joints per sheep, three replicate cultures per joint) of CPM per microgram of cellular DNA.

Animal studies using radioactively labelled HA have shown that it is rapidly cleared from the synovial cavity, largely via the lymphatics [64]. In an attempt to prolong the half-life of HA in the joint, cross-linked or otherwise modified HA preparations with markedly increased MW and resistance to degradation have been examined. This research has produced alternative treatment options for OA, which stay longer in the joint, and HA preparations with potentially different pharmacological properties [65]. HYADD4-G is a novel amide derivative of 500–730 kDa HA (Hyalgan®) where aliphatic amine (hexadecylamine) is bound to HA at the carboxylic group of the glucuronic acid (2% substitution). HYADD4-G has been observed to have superior rheological properties to Hyalgan® and to human SF [26]. Overall, both HA preparations in the current study have a beneficial effect on synovial pathological changes; however, the potential longer retention of HYADD4-G, which also induced increased HMW-HA and decreased intimal hyperplasia with fewer injections, may suggest potential advantages of this preparation.

There have been a number of reviews of the benefits of IA HA treatment in OA. One of the most recent systematic reviews of five HA trial meta-analyses (covering 11–37 studies each) concludes that this therapy results in modest improvement in validated outcomes [17], with four of the five meta-analyses rating HA as beneficial and safe. Contention arises, however, when attempts are made to explain how HA modulates the disease process, or which if any, of the various HA preparations are superior. It is clear from the present study that the actions of IA HA in OA synovium are multi-factorial. In the present animal model, IA HA (both Hyalgan® or HYADD4-G) reduced the overall pathology of synovia from meniscectomized joints. Neither of the HA preparations modified immunostaining of the six potential effector molecules examined. Further studies are necessary to elucidate the processes by which the different HA treatments are generating a positive effect in OA synovium.

**Rheumatology key messages**

- Increased synovial fibrosis and vascularity are pathological features of meniscectomy-induced OA.
- IA HA or its amide derivative administered in established disease reduced synovial fibrosis and vascularity.

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