Hyaluronic Acid Attenuates Osteoarthritis Development in the Anterior Cruciate Ligament-Transected Knee: Association with Excitatory Amino Acid Release in the Joint Dialysate

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ABSTRACT: We previously reported increased release of the excitatory amino acid (EAA) neurotransmitters, glutamate and aspartate, during the early stage of experimental osteoarthritis (OA). Our present objective was to study the effect of intraarticular injection of hyaluronic acid (HA) on OA development, and to analyze concomitant changes in EAA levels in dialysates of anterior cruciate ligament-transected (ACLT) knee joints. OA was induced in Wistar rats by ACLT of one hindlimb; the knee of the other hindlimb was used as the sham-operated control. HA group (n = 12) were injected intraarticularly in the ACLT knee with 1 mg of HA once a week for 5 consecutive weeks, starting at 8 weeks after surgery. Saline group (n = 12) were injected as above with normal saline. The sham-operated group underwent arthroty, but not ACLT, and received no treatment (n = 14). Twenty weeks after surgery, knee joint dialysates were collected by microdialysis and EAA levels assayed by high-performance liquid chromatography, and gross morphological examination and histopathological evaluation were performed on the medial femoral condyles and synovia. Rats receiving intraarticular HA injections showed a significantly lower degree of cartilage degeneration on the medial femoral condyle at both the macroscopic level and on the Mankin grading scale than rats receiving saline injections. Intraarticular HA treatment also suppressed synovitis. Moreover, glutamate and aspartate levels were significantly reduced in the HA group compared to the saline group. Intraarticular injection of HA limits articular cartilage and synovium damage and OA formation, and, in parallel, reduces EAA levels in ACLT joint dialysates. This study suggests that the underlying mechanism of the anti-inflammatory effect of HA is to inhibit glutamate and aspartate release in ACLT knee joints, which attenuates the early development of OA.

Keywords: excitatory amino acid; glutamate; osteoarthritis; hyaluronic acid; anterior cruciate ligament; microdialysis

INTRODUCTION

Osteoarthritis (OA), the most common cause of pain and disable in the elderly, is considered to be caused by multiple factors such as age, gender, genetic predisposition, mechanical stress, trauma, and weight.1,2 Studies had revealed a role of inflammatory process in the pathogenesis of OA,3 and potential mediators were described.4 Patients with rupture of the anterior cruciate ligament (ACL) develop posttraumatic OA of the knee.5 Restoration of knee stability by reconstruction provides symptomatic relief, but does not decrease...
the incidence of degenerative changes of the ACL injured knee. This suggests that the development of posttraumatic OA may not have a purely biomechanical origin, and that chemical changes may also be involved.

Hyaluronic acid (HA) is one of the major glycosaminoglycans of the extracellular matrix, in which it binds proteoglycans and provides intraarticular lubrication. It is also responsible for the viscoelastic properties of synovial fluid. In normal human synovial fluid, HA has an average molecular weight of 4–5 million Daltons, and is found at a concentration of 2.5–4 mg/mL, whereas, in OA synovial fluid, the concentration and molecular weight of the HA are reduced. Studies on animals have shown that HA effectively reduces articular pain and slows down the degenerative process of OA. Clinical trials and practice have demonstrated that intraarticular HA injection effectively relieves pain and improves motility function.

In vitro studies have shown that HA has a wide range of effects on chondrocytes, synoviocytes, and inflammatory cells in the synovial cavity of OA joints. HA can modulate inflammation by reducing neutrophil chemotaxis, macrophage proliferation, phagocytosis, and angiogenesis. It is speculated that it may also bind inflammatory mediators and free radicals, and remove them from the joint space via the lymphatics, thereby reducing cartilage damage. Intraarticular HA therapy, used for the treatment of pain associated with OA of the knee, has an anti-inflammatory effect and reduces pain by acting directly on cell receptors. Several cell types have been shown to have HA receptors, such as CD44 and intercellular adhesion molecule-1. Inhibiting HA binding to CD44 abrogates tissue edema and leukocyte infiltration in a murine arthritis model. Thus, the event triggered via the cell HA receptor may also be responsible for the observed anti-inflammatory activities of HA. In addition, it may have a direct analgesic effect on intraarticular nociceptors.

Previous studies in animals have demonstrated increased levels of glutamate and aspartate in the joint fluid in active arthropathies and that direct intraarticular injection of glutamate results in an increase in joint blood flow. Involvement of EAA in peripheral nociceptive transduction has also been postulated in animal models of acute arthritis. A potential peripheral action of glutamate has been suggested, as increased glutamate levels are seen in the peripheral tissues in acute and chronic inflammation in both animals and humans. Increased glutamate levels in the knee joint and medial articular nerve are seen after induction of arthritis in the rat. We previously reported that glutamate and aspartate levels were significantly increased in dialysates of anterior cruciate ligament-transected (ACLT) knees, suggesting a role of EAA in early OA development. Glutamate transporters have been identified in bone, raising the possibility that EAA participate in signal transduction between bone cells. Intraarticular HA has been shown to be clinically effective in reducing and delaying the development of OA, and is presently used in the treatment of arthropathy. The effect of HA on EAA production has not been examined. In this study, we investigated the effects of HA on the development of OA and on intraarticular EAA levels using a microdialysis technique that allows biochemical changes in joint dialysates to be examined continuously. HA was found to alleviate the gross morphological and histopathological changes seen in this OA model, and a significant decrease in glutamate and aspartate levels was seen in dialysates of HA-treated ACLT knee joints compared to those from saline-treated ACLT knees. We suggest that one of the mechanisms of the therapeutic effects of HA on OA is to attenuate the release of glutamate and aspartate.

MATERIALS AND METHODS

Animal Model

The experimental protocol was approved by the Animal Care and Use Committee of our Institute, and conformed to the National Institutes of Health guidelines for the care and use of animals in research. Thirty-eight 3-month-old male Wistar rats (body weight 260–290 g) were used and maintained under climate-controlled conditions on a 12-h light:12-h dark cycle at 22–24 °C and a relative humidity of 50–55%. The rats were allowed free access to standard rat chow and water. All experiments were performed during the light period. The animals were first placed for more than 30 min in long steel cages, allowing clear observation of their feet, and any rats with anatomical or gait abnormalities were excluded. The knee to undergo ACLT or sham operation was assigned randomly. The surgical procedure was modified from the protocol described by Stoop et al. The rats were anesthetized with 3% isoflurane in an oxygen/air mixture (1:1) at a flow rate of 3 L/min, anesthesia being considered adequate when no flexor withdrawal reflex was seen when the foot was pinched. Body temperature was monitored and maintained at 37 °C using a temperature controller (CMA 150, Bionalytical Systems Inc., USA) and a homeothermic blanket system. The knees were shaved and disinfected with betadine solution, then a medial parapatellar incision was made in the skin, and medial arthrotomy performed. The patella was dislocated laterally and the
knee placed in full flexion; then the ACL was exposed and identified visually and cut through the midsubstance. Adequacy of section was confirmed by a positive anterior draw sign. The joint cavity was washed with normal saline solution, the articular capsule sutured with 4-0 vicryl, and the skin closed with 4-0 nylon mattress sutures. The sham-operated group underwent arthrotomy, but not transection of the ACL. Cephazolin (20 mg/kg) was given intraperitoneally preoperatively and every 12 h for 3 days after operation for prophylactic infection control. The rats were allowed daily unrestricted cage activity after surgery.

**Experimental Design and HA Injection**

Eight weeks after surgery the animals were divided into three groups. Rats in the HA group \( (n = 12) \) were injected intraarticularly in the ACLT knee once a week for 5 weeks with 0.1 mL of highly purified HA (10 mg/mL in phosphate-buffered saline; molecular weight 800 kDa) from rooster comb (Artz, Seikagaku Corp., Tokyo, Japan), a protocol similar to that used clinically. Rats in the saline group \( (n = 12) \) were injected on the same schedule with 0.1 mL of sterile physiological normal saline. The sham-operated group \((n = 14)\) underwent arthrotomy, but not ACLT, and received no treatment. For intraarticular injection, rats were anesthetized with 3% isoflurane in an oxygen/air mixture (1:1) at a flow rate of 3 L/min, and injection performed under aseptic conditions by passing a 27-gauge needle attached to a tuberculin syringe through the joint capsule lateral to the patellar tendon. Several extension/flexions were performed after injection to ensure an equal distribution of the injected material throughout the joint cavity. All animals tolerated the injection procedure well, with no adverse reactions. At 20 weeks after ACLT, the microdialysis experiment was performed, then the animals were sacrificed and the knees examined histologically. The study design is summarized in Figure 1.

**Construction and Placement of the Microdialysis Probe**

At 20 weeks after ACLT or sham operation, a microdialysis probe was implanted in each knee joint under isoflurane anesthesia as described above. A 27-gauge needle attached to a tuberculin syringe was passed through the joint capsule lateral to the patellar tendon. Several extension/flexions were performed after injection to ensure an equal distribution of the injected material throughout the joint cavity. All animals tolerated the injection procedure well, with no adverse reactions. At 20 weeks after ACLT, the microdialysis experiment was performed, then the animals were sacrificed and the knees examined histologically. The study design is summarized in Figure 1.

**Measurement of Excitatory Amino Acids**

High-performance liquid chromatography with a fluorescence detector (model 126, Beckman Instruments Inc., Fullerton, CA) was used, as described in our previous report. In brief, a reverse-phase C-18 column with o-phthalaldehyde precolumn derivatization and a fluorescence detector was used. Samples were injected onto the column, which was eluted for 20 min at a flow rate of 0.45 mL/min with a linear gradient from 100% mobile phase A (20 mM sodium acetate, pH 7.2, containing 0.18% triethylamine, and 0.3% tetrahydrofuran) to 75% mobile phase A, 25% mobile phase B (100 mM sodium acetate/acetonitrile/methanol 1/2/2) in 30 min, then for 5 min with 100% mobile phase B. Using this protocol, glutamate, aspartate, serine, taurine, and glutamine were successfully separated. External standards containing 0, 10⁻⁸, 10⁻⁷, 10⁻⁶, or 10⁻⁵ M standard amino acids were run before and after each sample group. The detection sensitivity was 10⁻⁶ M. All standards and samples were analyzed in duplicate.

**Histopathological Examination of Joints**

The widths of the bilateral hindlimb knee joints were measured using calipers (AA847R, Aesculap, AG&CO,
described methods.33,34 Briefly, cartilage erosion was lesions of the medial femoral condyle using previously was examined for gross morphologic changes of cartilage treated with HA. Immediately after sacrifice, each knee in injections, or evaluations. All samples were examined who did not participate in any of the operations, and these numbers were used to identify the samples throughout the evaluation to prevent bias. All randomizations and numbering were performed by one assistant in the midsagittal plane, washed in running tap water, and paraffin-embedded in an automatic processor (Autotechnicon mono 2, Technicon Co., Chauncey, NY). Previous studies have demonstrated that the medial femoral condyles show the most advanced changes following ACLT,11,28 so these and the associated synovia were selected for histological preparation and assessment. Serial articular cartilage sections (5 μm) were cut on a Leica 2065 rotatory microtome (Leica Instruments, Wetzlar, Germany) from the central weight-bearing surface of the medial femoral condyles of the ACLT and sham-operated knees. Synovial membrane specimens were carefully dissected from the suprapatellar pouch and the medial tibiofemoral compartments and 10 slides prepared from each knee. Cartilage was stained with hematoxylin/eosin (H/E) and Safranin-O stain to assess general morphology and loss of proteoglycan in cartilage ground substance. All samples from all groups were numbered randomly, and these numbers were used to identify the samples throughout the evaluation to prevent bias. All randomizations and numbering were performed by one assistant who did not participate in any of the operations, injections, or evaluations. All samples were examined by two observers blinded as to which knee had been treated with HA. Immediately after sacrifice, each knee was examined for gross morphologic changes of cartilage lesions of the medial femoral condyle using previously described methods.33,34 Briefly, cartilage erosion was graded on a scale of 0–4, with 0 = normal; 1 = minimal fibrillation, or a slight yellowish discoloration of the surface; 2 = erosion extended to the superficial or middle layers; 3 = erosion extended to the deep layer; and 4 = erosion extended to the subchondral bone. Microscopic examination was used to grade the articular cartilage in the medial femoral condyles according to Mankin’s histologic grading;35 this score assesses structure (0–6 points), cellularity (0–3 points), matrix staining (0–4 points), and tidemark integrity (0–1 points), and has a maximum of 14 points. Scoring was based on the most severe histological changes seen in each cartilage section. The Mankin score was divided into three stages of 0–6 points (stage I; mild degenerative change), 7–9 points (stage II; moderate degenerative change), and 10 or more points (stage III; severe degenerative change, that is, cartilage disorganization or complete cartilage loss with extensive exposure of subchondral bone), a higher score indicating more severe damage. The synovium from the ACLT and sham-operated knees of each group was also processed, embedded in paraffin, and stained with H&E for cellular assessment by histology;36 this score assessed (1) synovial lining layer: hyperplasia of synovial lining cells (0–3 points), hypertrophy of synovial lining layer (0–3 points), and infiltration of inflammatory cells (0–3 points); (2) subsynovial tissue: proliferation of granulation tissue (0–3 points), vascularization (0–3 points), and infiltration of inflammatory cells (0–3 points), with a maximum of 18 points. The synovitis scores were divided into three stages: 0–6 points (mild synovitis), 7–12 points (moderate synovitis), and 13 or more points (severe synovitis). The higher the score, the more the damage.

Data and Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Data was analyzed using one-way ANOVA with Fisher’s post hoc tests for multiple comparisons. A p-value < 0.05 was considered significant.

RESULTS

At sacrifice, all ACLT knees showed complete transection of the ACL, with only a stump remaining. The surgical procedure (ACLT) in our present study was 100% successful. No signs of drug toxicity were noted in the group of rats treated with HA. The level of daily activity was similar in the rats from all three experimental groups, and there were no significant differences in body weight between the groups over the study period.

Knee Joint Width and Gross Morphologic Changes

As shown in Figure 2, at 20 weeks after surgery, the increase in width compared to week 0 in the saline, HA, and sham-operated groups was 2.6 ± 0.3, 1.0 ± 0.2, and 0.14 ± 0.13 mm, respectively, with a significant difference between the saline group and both the HA (p = 0.0008) and sham groups (p = 0.0005), and between the HA and sham groups (p = 0.042). The HA group showed a smaller increase in joint width than the saline group. In the saline and HA groups, the gross characteristics of cartilage degeneration of fibrillation, erosion, and ulcer formation, but not osteophyte formation, were seen in the medial femoral condyles. In the sham group, the cartilage on the medial femoral condyles was macroscopically normal, with a glistening, smooth surface, and no cartilage defects or osteophytes were observed, except for occasional fibrillation. Table 1 shows the gross evaluation scores for each group. A

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significant difference in gross morphologic score was found between the saline group (2.75 ± 0.13) and both the HA (1.33 ± 0.14, p = 0.004) and sham groups (0.25 ± 0.13, p = 0.0006) (Table 1), but not between the sham and HA groups (p = 0.39). The grade of cartilage damage in the HA group was significantly lower than that in the saline group, but not statistically different from that in the sham group. The ACLT knees treated with HA showed a marked reduction in the severity of lesions on the medial femoral condyles on gross examination (Table 1). Synovia from the saline group were hypertrophic, and showed a reddish yellow discoloration, while in the HA group they were thinner and the discoloration less intense. The synovia from the sham group had a white luster and transparent appearance., and showed no hyperemia or evidence of synovitis. Other quadrants of rat knee joints in the present study showed mild OA change (fibrillation, and mild decrease Safranin-O staining) in the medial tibial and lateral tibial plateau cartilage, and the lateral femoral condyles almost showed intact cartilage.

### Table 1. Macroscopic Evaluation of the Articular Cartilage of the Medial Femoral Condyles

<table>
<thead>
<tr>
<th>Group</th>
<th>Score</th>
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<tr>
<td>ACLT-S (n = 12)</td>
<td>2.75 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACLT-HA (n = 12)</td>
<td>1.33 ± 0.14&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sham (n = 12)</td>
<td>0.25 ± 0.13</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM.

<sup>a</sup>p < 0.05 compared to the sham group.

<sup>b</sup>p < 0.05 compared to the saline group. ACLT-S = ACLT treated with normal saline; ACLT-HA = ACLT treated with HA group; Sham = sham-operated group. Macroscopic grading = 0–4 (0 = surface appears normal; 1 = minimal fibrillation, or a slight yellowish discoloration of the surface; 2 = erosion extended to the superficial or middle layers; 3 = erosion extended to the deep layer; and 4 = erosion extended to the subchondral bone).

**Microscopic Findings**

On histopathological examination using H/E or Safranin-O staining, the cartilage on the medial femoral condyle in the ACLT knees of rats injected with saline or HA exhibited various degrees of pathological changes of OA. Representative histological results are shown in Figure 3. Specimens from the saline group had obvious morphologic changes, including loss of superficial cartilage layers, fibrillation and fissures extending to transitional and radial layers, and chondrocytes hypocellularity (Fig. 3A). In the HA group, there was a marked reduction in the severity of lesions of the medial femoral condyles; only fibrillation and fissure extended to superficial layer of cartilage was observed (Fig. 3B). The medial femoral condyles of most of the sham-operated group had a normal histological appearance (Fig. 3C) except in one rat, which showed a minimal loss of Safranin-O staining at the superficial layer and minimal irregularities of the cartilage surface (figure not shown). The Mankin grading is shown in Table 2. Significant differences were found between the sham group (0.83 ± 0.16) and both the saline (8.12 ± 0.27, p = 0.0003) and HA groups (3.33 ± 0.18, p = 0.008), and between the saline and HA groups (p = 0.005) (Table 2). The Mankin score for the HA group was significantly lower than that for the saline group. The synovia from the saline group were thick, had focal villi, and showed hyperplasia of the lining cells of the synovia and moderate infiltration of mononuclear cells (Fig. 3D). Synovial inflammation was less severe in the HA group than the saline group (Fig. 3E). The histology of the synovia from the sham-operated group was within normal limits (Fig. 3F). The synovitis scores by microscopic evaluation are shown in Table 2; significant differences were found between the sham group (1.72 ± 0.23) and both the saline (9.82 ± 0.28, p = 0.0003) and HA groups (4.21 ± 0.22, p = 0.008), and between the HA and saline groups (p = 0.006) (Table 2). The score for the synovial membrane in
Figure 3. Histopathological evaluation of the cartilage of the medial femoral condyles (Stain, H/E; original magnification, ×200) and synovium (H/E stain, original magnification, ×200). (A) ACLT specimen from saline group shows a decrease in cartilage thickness, disappearance of surface layer cells, fissure extend to the transitional and radial zones (arrow), and chondrocytes hypocellularity of the transitional and radial zones. (B) ACLT specimen from the HA group shows irregularity of the surface layer, fibrillation and fissure of superficial layer of cartilage (arrow), and little diffuse hypercellularity in the transitional and radial zones in H/E staining. (C) In the sham-operated group, a smooth superficial layer of the cartilage was observed (arrow), and cartilage matrix was consistently well stained with H/E. (D) Synovium in saline group shows synovial lining cell proliferation with a marked mixed cellular infiltration in the underlying tissues, consisting mainly of monocytes. Fibrinoid exudate and cartilage fragment (arrow) was also noted. (E) Synovial membrane from the HA group shows focal synovitis (arrow) with a mild mononuclear cell infiltrate. (F) Synovial membrane from a sham-operated knee shows no synovial lining cell hyperplasia (scale bar = 100 μm).
the ACLT knee was lower in the HA group than in the saline group.

Excitatory Amino Acid Levels in Knee Joint Dialysates

EAA levels were measured in the HA- and saline-treated ACLT knees and the sham-operated knees at 20 weeks postsurgery, the mean resting concentration of glutamate and aspartate in synovial fluid’s dialysates of the contralateral sham rats were 1.98 ± 0.25 and 0.35 ± 0.03 µM (mean ± SEM), respectively. The EAA levels in the contralateral sham knee being taken as 100%. Significant differences in glutamate concentration were found between the saline group (196 ± 17%) and both the sham (101 ± 1.32%, $p = 0.0005$) and HA groups (131.2 ± 10.0%, $p = 0.006$), but not between the HA and sham groups ($p = 0.339$) (Fig. 4A). For aspartate, significant differences were again found between the saline group (189.2 ± 13.2%) and both the sham (104.0 ± 2.1%, $p = 0.0007$) and HA groups (126.4 ± 13.0%, $p = 0.003$), but not between the HA and sham groups ($p = 0.469$) (Fig. 4B). Glutamate and aspartate levels in the HA group were significant lower than those in the saline group. There were no differences in serine, taurine, and glutamine levels in the dialysates between the HA- and saline-treated ACLT knees (data not shown).

DISCUSSION

This is the first study to demonstrate that intraarticular HA treatment reduces glutamate and aspartate levels in ACLT joint dialysates at 20 weeks after surgery. Knee instability following a complete tear of the ACL often induces OA accompanied by degradation of the articular cartilage matrix. $^{5,6}$ Animal studies are essential, as the early biochemical and cellular changes of OA are almost impossible to study in the natural disease because the time of onset is not usually known; in addition, a control knee is available in the same animal, eliminating individual variation. The ACLT model has proved a useful tool in the investigation of OA development, because the biochemical and pathological changes are identical.

Table 2. Histological Evaluation of the Articular Cartilage of the Medial Femoral Condyles and Synovial Tissue

<table>
<thead>
<tr>
<th>Group</th>
<th>Osteoarthritic Score</th>
<th>Synovitis Score</th>
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<tr>
<td>ACLT-S ($n=12$)</td>
<td>8.12 ± 0.27$^a$</td>
<td>9.82 ± 0.28$^a$</td>
</tr>
<tr>
<td>ACLT-HA ($n=12$)</td>
<td>3.33 ± 0.18$^{a,b}$</td>
<td>4.21 ± 0.22$^{a,b}$</td>
</tr>
<tr>
<td>Sham ($n=12$)</td>
<td>0.83 ± 0.16</td>
<td>1.72 ± 0.23</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SEM. $^a p < 0.05$ compared to the sham group. $^b p < 0.05$ compared to the saline group. ACLT-S = ACLT treated with normal saline; ACLT-HA = ACLT treated with HA group; Sham = sham-operated group. For the osteoarthritic score (Mankin score) and synovitis score, refer to the Materials and Methods.
to those seen in human OA. The lesion site, as in humans, is mainly on the medial femoral condyle, and a comparison can be made before and after intervention.\textsuperscript{11,28}

Intraarticular HA treatment of the knee in patients with OA reduces painful symptoms and improves general activities of daily living and joint mobility.\textsuperscript{12,30,37} Synovial fluid from arthritic joints contains lower concentration of HA than that from normal joints.\textsuperscript{7} A decrease in HA levels in the synovial fluid may result in damage to cartilage surface and increase the permeability of cartilage, allowing proteins and other high molecular weight substances to penetrate into the cartilage matrix.\textsuperscript{36} Intraarticularly injected HA binds free proteoglycan in the cartilage matrix, and inhibits further proteoglycan release, thus protecting the cartilage matrix and cells.\textsuperscript{7,9,18} HA does not infiltrate normal cartilage.\textsuperscript{36} Exogenous HA is known to stimulate endogenous HA production by positive feedback.\textsuperscript{30} It is speculated that HA may bind inflammatory mediators and free radicals, removing them from the joint space via the lymphatics, thereby reducing cartilage damage.\textsuperscript{18} In our present study, both the macroscopic grading score and the Mankin score were significantly lower in the HA group than in the saline group (Fig. 3 and Tables 1 and 2), mainly in terms of a reduction in the severity of structural changes and loss of Safranin-O staining. HA injection significantly reduced the severity of cartilage degradation in the ACLT knee. The sham-operated knees did not receive the same injection (saline) or HA as the ACLT knees in our present study, but this does not detract from the important differences observed between ACLT treated with HA and those injected with saline. In our present study, the gross characteristics showed absence of osteophyte formation in ACLT knees with saline injection. This is somewhat different from other animal models with osteophyte formation.\textsuperscript{31,38,39} The possible reasons for the difference might be due to: (1) different animals were used: Wistar rats were used in our study; other studies were dogs, rabbits, or sheep; (2) space for daily activity: in our study, two rats were put in one cage, a narrow space for daily activity (weight-bearing) after surgery; (3) duration after ACLT for histology examinations: 20 weeks (our study) versus 32 weeks or 54 months (in other studies). The Mankin scores reported in the ACLT-saline treated group in Table 2 showed 8.12 are two points higher than those reported in our previous report (scores = 5.93),\textsuperscript{28} whereas the sham-operated knees were slightly lower in the present study (0.83 vs. 1.35). In our present study, after ACLT, two rats were put in one cage, while in our previous study; three rats were put in one cage. The daily activity (weight-bearing) in the present study might be increased; therefore, with severer Mankin’s scores (8.12) when compared to our previous study (5.93). In OA, synovial inflammation was also reported to play an important role in the disease process.\textsuperscript{40} In our present study, the synovitis score was lower in the HA group than in the saline group (Fig. 3 and Table 2), mainly in terms of a reduction in the intensity of villous hyperplasia and mononuclear infiltration. The improved Mankin score and synovitis score in the HA-treated ACLT knees in our study are in agreement with previous reports.\textsuperscript{11,13}

An important finding of the present study was the significant decrease in glutamate and aspartate concentrations in the joint dialysate of HA-treated ACLT knees. Glutamate is the major excitatory amino acid in the central nervous system (CNS). A recent report suggested a possible role of glutamate as a therapeutic target in neurodegeneration disease.\textsuperscript{41} Intraarticular EAA injection resulted in thermal hyperalgesia and mechanical allodynia, which were attenuated by local injection of N-methyl-D-aspartate (NMDA) or non-NMDA receptor antagonists.\textsuperscript{27} Lawand et al.\textsuperscript{26} also demonstrated that intraarticular lidocaine injection prevented glutamate release and nociceptors sensitization of inflammatory joints. Moreover, in inflamed arthritic knees, an increase of glutamate concentration was observed not only in the axons in the inflamed region,\textsuperscript{25} but also in the synovial fluid.\textsuperscript{23} Glutamate and glutamate receptor agonists was also reported to induce tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) production in synovial cells, which further upregulated cytokines and chemokines production in rheumatoid arthritis patients.\textsuperscript{42} The HA injection in the present study might be attenuated the mechanical stimuli-induced EAA release and then the down stream inflammatory responses.

Glutamate transporters were identified involving in osteogenic signalling,\textsuperscript{29} and vesicular glutamate transporters were demonstrated constitutively expressed in cultured osteoblasts and osteoeclasts.\textsuperscript{53,44} Furthermore, glutamate was demonstrated to mediate intercellular communication in bone cells, in a manner similar to the synaptic transmission in the CNS, it contributes to bone matrix regulation.\textsuperscript{45,46} Constitutive release of glutamate was demonstrated in cultured calvarial rat osteoblasts via a voltage-dependent calcium entry mechanism.\textsuperscript{43} The expression of NMDA receptors in human and rat osteoblasts and osteoclasts suggests a role of glutamate in bone
cells signalling.\(^{37}\) Immunohistochemical evidence also demonstrated glutamate-containing nerves in bone.\(^{48}\) Moreover, using immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR) techniques, NMDAR1 and NMDAR2A subunits of NMDA receptors are both expressed in normal and OA chondrocytes.\(^{49}\) Furthermore, both metabotropic and ionotropic glutamate receptors have been shown to be functional in osteoblasts\(^{50}\) and osteoclasts,\(^{51}\) and antagonists to these receptors can modify the phenotype of bone cells.\(^{51,52}\) Intraarticular HA has also been demonstrated reduced PGE2 and cyclic AMP levels in the synovial fluid of OA patients; HA attenuates the inflammatory process and relieves pain by inhibiting articular chondrocytes PGE2 production.\(^{14}\) Moreover, the viscosupplementation effect of HA may also be sensory afferent fibers and nociceptors in synovial and subsynovial tissues.\(^{7,9}\) Injection of HA into the inflamed knee joint was also found reduced inflammation-evoked nerve discharges in cats.\(^{53}\) Furthermore, high molecular weight HA was demonstrated to activate the primary human osteoblasts from OA subchondral bone by modifying their biological synthetic capacity.\(^{54}\) Despite the short half-life of HA in the joint, however, it seems that the effect of high molecular weight HA injection may persist for several weeks or even months after intraarticular injection;\(^{37}\) therefore, the effects of HA in the joint could be related to its pharmacologic effects rather than its mechanical property of increasing viscosity. The present study is the first to demonstrate that intrarticular HA injection reduces glutamate and aspartate concentration in ACLT knees. This attenuation of EAA level by HA may slow down the pathological process of OA in the ACLT knee; however, the etiopathologic relationship still needs further investigation.

In conclusion, our results show that the protective effects of HA on degenerative changes in the cartilage and synovitis are associated with a reduction in glutamate and aspartate levels, at least in part, in the ACLT knee joint. These findings may pave the way for further investigations of EAA as a potentially therapeutic target for the treatment of the inflammatory component in early OA.

ACKNOWLEDGMENTS

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