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学位論文

A study of the chondroprotective effects of high molecular weight cross-linked hyaluronic acid in a rabbit knee osteoarthritis model

(家兎関節症モデルにおける高分子ヒアルロン酸
の軟骨保護作用に関する研究)

2015年3月

北海道大学

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ABSTRACT

Objectives:

Recent reports show the efficacy of high-molecular-weight (MW) hyaluronic acid (HA) in treating osteoarthritis. However, its mechanism remains unclear. In this study, I examined the histopathological changes and friction coefficients in osteoarthritic knee joints after injecting high-MW cross-linked (CL) HA.

Design:

A bilateral anterior cruciate ligament transection model in Japanese white rabbits was used ($n = 20$). From week 5 after transection, low-MW HA (0.8×10^6 Da; HA80) or high-MW CL HA (6×10^6 Da; HA600) was injected weekly into the right knee for threeweeks; normal saline (NS) was injected into the left knee ($n = 10$ in each group). A bilateral sham operation was undertaken to exclude spontaneous osteoarthritis ($n = 3$). Results were evaluated with macroscopy (India ink), histopathology (Kikuchi's score), biomechanical testing, and rheological assessment of the joint fluid viscoelasticity. Statistical analysis was performed using one-way analysis of variance with a 95% confidence interval ($P < 0.05$).

Results:

The macroscopic findings showed severely damaged cartilage in 30% of the NS group and 20% of the HA80 and HA600 groups and intact cartilage in 100% of the sham group. The histological scores and friction coefficients of the HA600 group were significantly lower than those of the NS group ($P = 0.007$ and $P = 0.002$, respectively). Viscoelasticity measurements of the joint fluid showed no significant differences between the three groups.

Conclusion:

High-MW CL HA exerts potential chondroprotective effects and produces superior friction coefficients. Our results suggest that HA600 delays the progression of osteoarthritis effectively and improves joint lubrication significantly.

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List of Publications and Presentations at Academic Meetings

I. Publications:

- 1) Title: Chondroprotective effects of high-molecular-weight cross-linked hyaluronic acid in a rabbit knee osteoarthritis model.

Authors: Sameh Elmorsy, Tadanao Funakoshi, Fumio Sasazawa, Masahiro Todoh, Shigeru Tadano, Norimasa Iwasaki..

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II. Presentations at academic seminars

- 1) 2013 Orthopaedic Research Society Annual Meeting

January 2013, San Antonio, USA.

Poster presentation

- 2) 26th Annual Meeting of the Japanese Society of Cartilage Metabolism

March 2013, Osaka.

Oral presentation

- 3) 86th Annual meeting of the Japanese Orthopedic Association

May 2013, Hiroshima

Oral presentation

- 4) 11th International Cartilage Repair Society (ICRS) Congress

September 2013, Izmir, Turkey

Poster presentation

- 5) 2014 Orthopaedic Research Society Annual Meeting

March 2014, New Orleans, USA

Poster Presentation

- 6) 86th Annual meeting of the Japanese Orthopedic Association
May 2013, Hiroshima
Oral presentation

- 7) 29th Annual research meeting of the Japanese Orthopaedic Association
October 2014, Kagoshima
Poster presentation

- 8) 26th SICOT Triennial World Congress
November 2014, Rio de Janeiro, Brazil
Oral Presentation

Introduction

Osteoarthritis (OA) is the clinical and pathological outcome of functional failure of synovial joints. It is characterized by joint pain and loss of function in the absence of chronic autoimmune or autoinflammatory mechanisms. The prevalence of OA increases with age, and the disorder accounts for a massive socioeconomic challenges, with millions of people affected worldwide. Aging of the population, coupled with epidemic increases in other risk factors such as obesity, suggests that the impact on society of OA-linked problems will continue to rise. From a clinical point of view, single or multiple joints could be affected, and the severities of pain and loss of function varies greatly between patients ¹⁻⁵.

Added to the involvement of several joint tissues, OA featured by the inability to repair the damaged cartilage due to biomechanical and biochemical changes in the joint. Cartilage is avascular, which limits the supply of nutrients and oxygen to the chondrocytes—the cells that are responsible for the upkeep of extracellular matrix. At early stages of OA, in attempting a repair process, clusters of chondrocytes form in the damaged areas and the concentration of growth factors in the matrix increases ^{6,7}. This attempt subsequently fails and leads to an imbalance in favour of degradation. Increased synthesis of tissue-destructive proteinases (matrix metalloproteinases and aggrecanases), increased apoptotic death of chondrocytes, and inadequate synthesis of components of the extracellular matrix, lead to the formation of a matrix that is unable to withstand normal mechanical stresses ^{8,9}. Consequently, the tissue enters a vicious cycle in which breakdown dominates synthesis of extracellular matrix. Since articular cartilage is aneural, these changes do not produce clinical signs unless innervated tissues become involved. This justifies the late diagnosis of OA ¹⁰.

Despite the pathophysiology of OA has been linked to cartilage, a recent report showed an additional and integrated role of bone and synovial tissue, and patchy chronic synovitis is evident in the disease ¹¹. Synovial inflammation underlies to clinical symptoms such as joint swelling and inflammatory pain, and it is thought to be secondary to cartilage debris and catabolic mediators entering the synovial cavity. Catabolic and pro-inflammatory mediators and inflammation which are the by-products of synovial macrophages, negatively affect the balance of cartilage matrix degradation and repair ¹². In turn this increases the synovial

inflammation which happens in both early and late phases of OA, generating a vicious cycle¹⁰.

In OA, osteophyte formation, bone remodeling, subchondral sclerosis, and attrition are crucial for radiological diagnosis. These changes take place during the final stage of the disease, and also at the onset of the disease—possibly before cartilage degradation. This finding led to the suggestion that subchondral bone could initiate cartilage damage^{10,13,14}.

Current treatments for OA range from minimal nonsurgical measures to invasive and surgical modalities. Nonsurgical nonpharmacological measures include exercise, weight loss, and bracing; surgical measures vary from arthroscopic debridement and osteotomy to arthroplasty. Pharmacological therapies, such as analgesics, intra-articular injections, and topical treatments, mainly target the palliation of pain^{15,16}. Chondroprotective agents are defined as compounds that (1) stimulate the synthesis of collagen and proteoglycans by chondrocytes and production of hyaluronic acid (HA) by synoviocytes; (2) inhibit cartilage degradation; and (3) prevent fibrin formation in the subchondral and synovial vasculature¹⁷. Compounds that show some of these characteristics are endogenous molecules of the articular cartilage, including HA, glucosamine, and chondroitin sulfate.

Viscosupplementation is an intra-articular therapeutic modality based on the physiological importance of HA in the synovial joints^{18,19}. HA is a heteropolysaccharide formed by a variable number of repeating units of D-glucuronic acid and N-acetylglucosamine. It is formed by synoviocytes, fibroblasts, and chondrocytes inside joints and is present in the synovial fluid and the extracellular matrix of the cartilage. HA is crucial to the viscoelastic properties that allow the efficient movement of articular joints²⁰⁻²³. HA is an abundant component of synovial fluid. Also it provides a backbone upon which proteoglycans can bind or aggregate to form a macromolecule with a weight up to 200 million daltons²⁴ (Fig. 1²⁵). Articular cartilage is composed mainly of water (70%-80%) which shifts in and out of cartilage to allow surface deformation as a consequence to different stress loads (Table 1²⁶). Proteoglycan concentration and water content vary through the depth of the articular cartilage tissue. Near to the articular surface, proteoglycan concentration is relatively low, while the water concentration is the highest. In the deeper areas of the cartilage, close to

the subchondral bone, the proteoglycan concentration is highest, and the water content is the lowest^{27,28}. Collagen is a fibrous protein that makes up 60 to 70% of the dry weight of the tissue. Type II collagen is the predominant type in the articular cartilage, although other types are present in smaller amounts²⁹. The structure of articular cartilage is described in terms of four zones between the articular surface and the subchondral bone: the surface or superficial tangential zone, the intermediate or middle zone, the deep or radiate zone, and the calcified zone (Fig. 2³⁰). The calcified cartilage is the boundary between the cartilage and the underlying subchondral bone. The interface between the deep zone and calcified cartilage is known as the tidemark³¹.

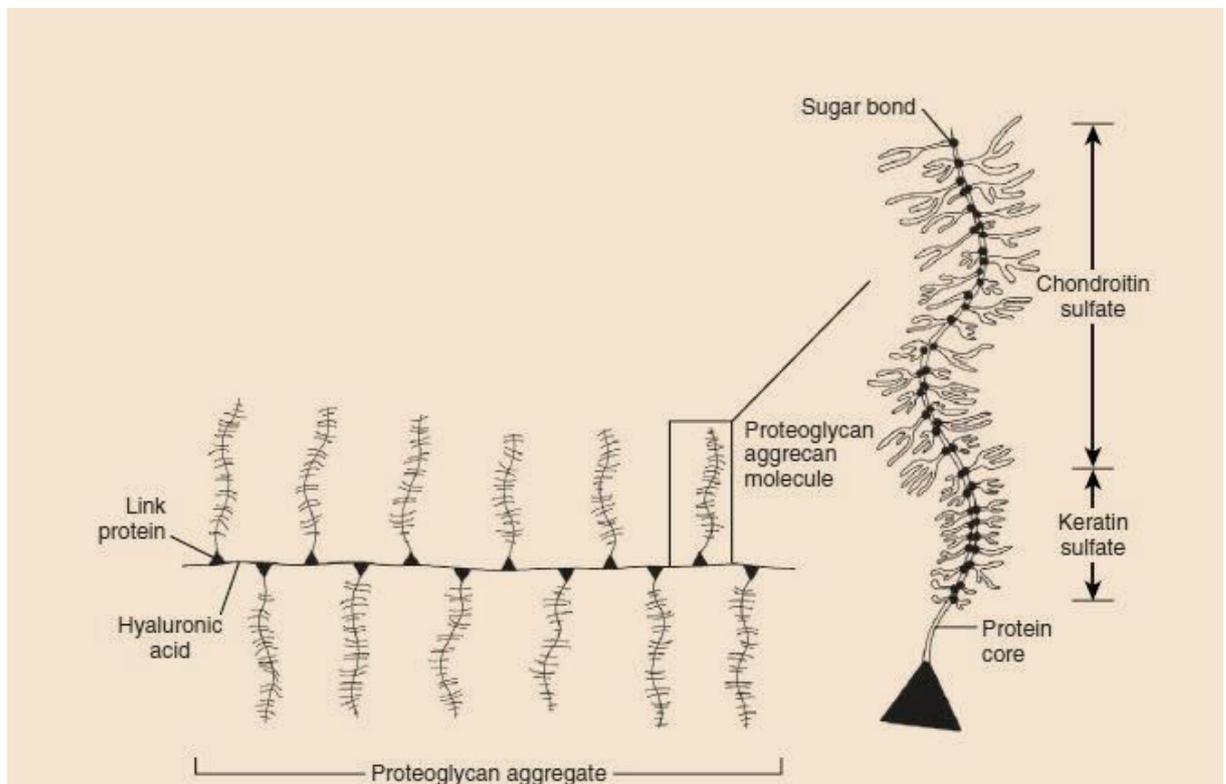


Figure 1 . Proteoglycan aggregates and aggrecan molecule²⁵.

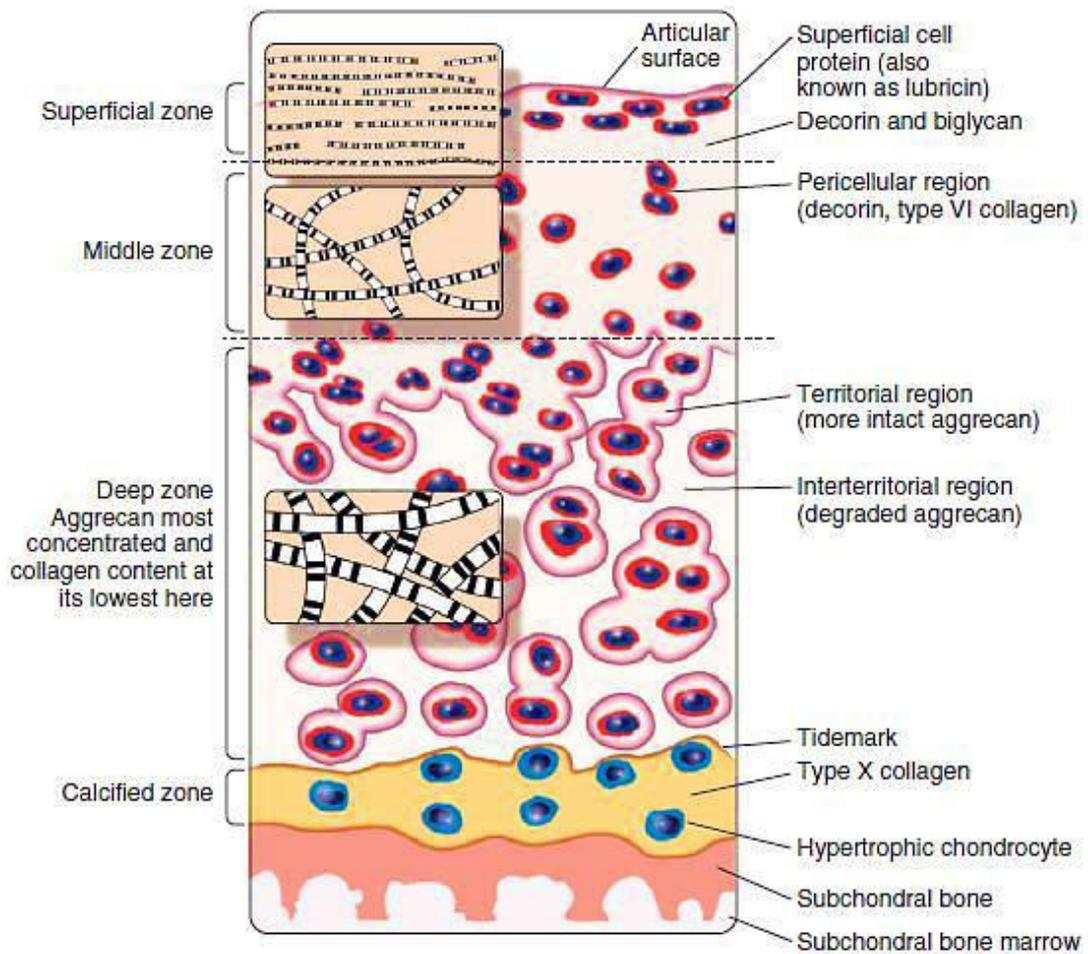


Figure 2. The structure of human adult articular cartilage. Insets show the relative diameters and orientations of collagen fibrils in the different zones ³⁰.

Table I. Biochemical changes of articular cartilage²⁶

Parameter	Effect of Aging	Effect of OA
Water content (hydration; permeability)	↓	↑
Collagen	Content remains relatively unchanged	Becomes disorderly (breakdown of matrix framework) Content ↓ in severe osteoarthritis Relative concentration ↑ (because of loss of proteoglycans) Collagen type VI content ↑
Proteoglycan content (concentration)	↓ (Also, the length of the protein core and glycosaminoglycan chains decreases)	↓
Proteoglycan synthesis	↓	↑
Proteoglycan degradation	↓	↑↑↑
Chondroitin sulfate concentration (includes both chondroitin-4- and -6-sulfate)	↓	↑
Chondroitin-4-sulfate concentration	↓	↑
Keratin sulfate concentration	↑	↓
Chondrocyte size	↑	
Chondrocyte number	↓	
Modulus of elasticity	↑	↓

Several HA preparations are being marketed with varying molecular weights (MWs), ranging from 0.5×10^6 Da to 6×10^6 Da. Clinical trials have not indicated a clear advantage of one product over another³². One of the goals of HA therapy is to restore the viscoelasticity of the synovial fluid and the natural protective functions of HA in the joint. In humans, the MW of HA in a healthy knee joint is 6×10^6 Da. It has been reported that the MW of HA in a human arthritic knee joint decreases to $0.5\text{--}3 \times 10^6$ Da³³. HA can potentially improve viscoelasticity, and a higher MW of HA is thought to improve the viscoelastic properties of HA preparations. In the American Academy of Orthopaedic Surgeons (AAOS) clinical practice guideline, “Treatment of Osteoarthritis of the knee”, HA injection is not strongly recommended because in the adopted studies none of the improvements met the minimum clinically important improvement thresholds meanwhile the guideline reported that most of the statistically significant outcomes were associated with high-molecular cross linked hyaluronic acid³⁴. Referring to Osteoarthritis Research Society International (OARSI) guidelines which were adopted by the Japanese Orthopaedic Association (JOA) the recommendation level remains uncertain³⁵. This difference in recommendations might be due to the different studies adopted for each guideline, moreover it strengthen the need for more research to elaborate the mechanisms and the effects of HA injection. Also, the effects of high-MW cross-linked (CL) HA on joint viscoelasticity and joint lubrication have not yet been reported.

Hypothesis

I hypothesized that high-MW CL HA improves joint lubrication and has an enhanced chondroprotective effect compared with lower-MW HA.

Objectives

This study aimed to examine the histopathological changes in osteoarthritic knee joints after HA injection and to investigate the relationship between the friction coefficient and the MW of HA.

Abbreviations List

AAOS	American Academy of Orthopaedic Surgeons
ACL	Anterior Cruciate Ligament Transection
ANOVA	Analysis of variants
CI	Confidence Interval
CL	Cross Linked
DMA	Dynamic Mechanical Analyzer
Da	Dalton
HA	Hyaluronic acid
JOA	Japanese Orthopaedic Association
MW	Molecular Weight
NS	Normal Saline
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
SD	Standard Deviation

Materials and methods

Osteoarthritis model

Twenty-three female Japanese white rabbits aged 12 weeks and weighing 2.6–2.9 kg were purchased from a professional breeding company (Japan SLC Inc., Hamamatsu, Japan) and used for this experiment. Our institutional Animal Care Committee approved the study, which was conducted according to its ethical guidelines and regulations. All animals were anesthetized with an intravenous injection of 0.05 mg/kg phenobarbital sodium, followed by gas anesthesia with isoflurane. Both knees of each rabbit were shaved, prepared, and draped in a sterile environment. To generate osteoarthritic joints, anterior cruciate ligament transection (ACLT) was performed in both knees using a medial parapatellar approach. After the patella was dislocated laterally, the knee was flexed maximally so that the anterior cruciate ligament could be readily visualized and identified. It was then transected with a #11 blade. The knee joints were inspected for bleeding and washed thoroughly with sterile normal saline (NS). The joint capsule was closed with a running suture of 4-0 nylon, and the skin incision was closed with running mattress sutures of 3-0 nylon³⁶. Instability was confirmed in all knees with positive anterior drawer and Lachman tests. After surgery, the animals were allowed unrestrained cage activities while they were monitored for infections and other complications. To rule out the development of spontaneous OA, a bilateral arthrotomy without the ACLT was performed as a sham non-OA model.

Injection materials

In this study, we used two HA preparations of different MWs: HA80 (Artz Dispo®, Seikagaku Corp., Tokyo, Japan), with an average MW of 0.8×10^6 Da; and HA600 (Hylan G-F 20, Synvisc®, Genzyme, a Sanofi company, Cambridge, MA), with an average MW of 6×10^6 Da. Both are available for patient use as a medical device loaded with sterile injection material. We used NS (0.9%) as the control.

Treatment regimens

In postoperative week 5, 46 knees from 23 rabbits were injected with NS or HA (HA80 or HA600). NS was injected into the left knees and HA (HA80 or HA600) was injected into the right knees. The sham group (6 knees from 3 rabbits) received bilateral NS injections. The intra-articular injection of 0.3 mL of each material was performed under intravenous anesthesia induced with 0.05 mg/kg phenobarbital sodium using a 26-gauge syringe under sterile conditions. Three weekly injections were administered, and all animals were killed 8 weeks after surgery (1 week after the third injection) with an intravenous lethal dose of phenobarbital sodium. From the OA groups, thirty knees were randomly assigned to one of three treatment groups (10 per group) with NS, HA80, or HA600. From the sham group, 5 knees were collected randomly from a total of 6 knees for evaluation.

Macroscopic evaluation

Macroscopic assessment of the knees (5 knees per group) was performed in all four compartments of every knee: medial femoral condyle, lateral femoral condyle, medial tibial plateau, and lateral tibial plateau. A solution of India ink diluted with phosphate-buffered saline (1:5) was used to stain the articular cartilage, and the stained specimens were photographed using a high-resolution digital camera. The findings were evaluated according to the following classification: Grade 1 (intact surface), surface was normal in appearance and did not retain India ink; Grade 2 (minimal fibrillation), surface retained India ink as elongated specks or light-gray patches; Grade 3 (overt fibrillation), areas were velvety in appearance and retained India ink as intense black patches; and Grade 4 (erosion), loss of cartilage exposing the underlying bone. Grade 4 was divided further into the following three subgrades: Grade 4a, erosion <2 mm; Grade 4b, erosion \geq 2 mm and < 5 mm; and Grade 4c, erosion \geq 5 mm³⁷.

Histopathological evaluation

The distal femur and proximal tibia from each knee (5 knees per group) were fixed with 4% phosphate-buffered paraformaldehyde for 24 hours, decalcified with 10%

ethylenediaminetetraacetic acid (pH 7.4) for 10 days, and then embedded in paraffin. A 5 μ m thick sagittal section was cut from the center of each medial femoral condyle. The sections were stained with Safranin-O. The slides were coded before microscopic examination and evaluated by three observers. Degenerative changes to the articular cartilage were assessed quantitatively using the scoring system described by Kikuchi *et al.* (Table II)³⁸

Table II. Histopathological scores for the evaluation of cartilage degeneration³⁸

Category	+1	+2	+3	+4
Loss of superficial layer	<Slight	Moderate	Focally severe	Extensively severe
Erosion of cartilage	<Detectable	Moderate	Focally severe	Extensively severe
Fibrillation and/or fissures	<Noticeable (<1 very small)	Moderate (1 small)	Marked (2 small or 1 medium)	Extensive (3 small, 2 medium, or 1 large)
Loss of proteoglycans	<Paler stain than control	Moderate loss of Safranin-O stain	Marked loss of Safranin-O stain	Total loss of Safranin-O stain
Disorganization of chondrocytes	Noticeable	Moderate, with some loss of columns	Marked loss of columns	No recognizable organization
Loss of chondrocytes	<Noticeable reduction in cells	Moderate reduction in cells	Marked reduction in cells	Very extensive reduction in cells
Exposure of subchondral bone	<Focal exposure of bone	Moderate exposure of bone	Fairly extensive exposure of bone	Very extensive exposure of bone
Cluster formation	<3–4 small or 1–2 medium	5–6 small, 3–4 medium, or 1–2 large	7 or more medium or 5–6 large	7 or more small, 5–6 medium, or 3–4 large

Friction test

Fifteen knees from the OA groups were used for this test (5 per group), and these specimens were used exclusively for this test. The effects of the intra-articular injection of the test substances on joint lubrication were assessed using a pendulum friction tester designed by our laboratory for small samples (Fig.3). The knees were resected at the proximal end of the femoral shaft and at the distal end of the tibia and then secured to polyethylene tubes with bone cement. All soft tissues were removed from the joint except the joint capsule and the tendons and ligaments around the knee. The distal end of the tibia of each sample joint was attached to the base plate, and the femoral shaft was attached to the pendulum. The pendulum motion was calculated from two translational displacements by laser displacement sensors (LK-G30, Keyence, Tokyo, Japan), and the angular displacement was calculated by an accelerometer that detected the direction of gravity (100 Hz) (WAA-006, Wireless Technologies, Inc., Tokyo, Japan). The total weight of the pendulum, including its frame, was 40 N in normal sea-level gravity. During the experiments, the joints were kept moist with injections of NS. Based on the linear damping oscillation curve of the pendulum, the frictional coefficient μ was calculated using the following equation: $\mu = L\Delta\theta/4r$, where r is the radius of the rabbit femoral condyle, L is the distance between the pendulum's center of gravity and center of rotation, and $\Delta\theta$ is the average decrease in the amplitude of the pendulum swing per cycle.

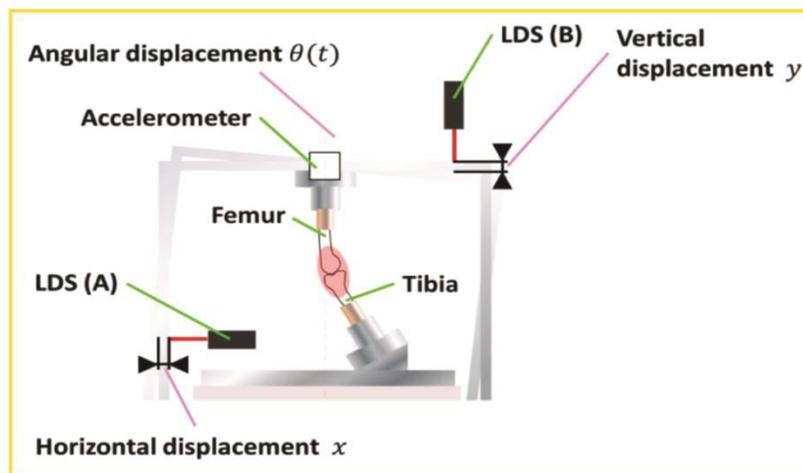
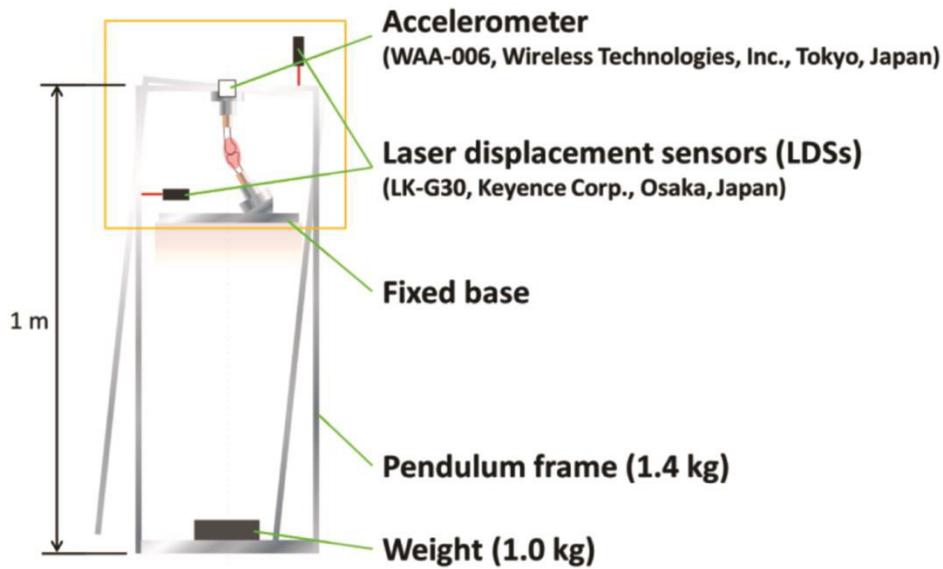


Figure 3. Illustration of the pendulum friction tester used in this study. The friction coefficient μ was calculated using the equation $\mu = L\Delta\theta/4r$, where r is the radius of the rabbit femoral condyle, L is the distance between the pendulum's center of gravity and center of rotation, and $\Delta\theta$ is the average decrease in the amplitude of the pendulum swing per cycle

Dynamic mechanical analysis

After the animals were killed, the joint fluid was aspirated and collected. The samples were centrifuged and stored without preservatives at $-80\text{ }^{\circ}\text{C}$. Before testing, the samples were thawed to room temperature. The viscoelastic properties of the joint fluids were measured using a dynamic mechanical analyzer (DMA) (Rheosol-G3000NT, UBM Co. Ltd, Huga, Japan). The rheological properties of the synovial fluids were evaluated at a controlled temperature of $25 \pm 1\text{ }^{\circ}\text{C}$. The DMA has two plates, one parallel and one cone-shaped, and the sample was placed between them. The synovial fluid material was subjected to sinusoidal shear forces (strain, stress), and the output signals were recorded. The rheology of the synovial fluid samples was investigated by measuring the steady-state viscosity and small-amplitude oscillatory movements. The small-amplitude oscillatory shear experiments allow the measurement of the unsteady response of a sample and hence the determination of its linear viscoelastic properties^{39,40}. The data collected from the DMA are analyzed to yield the shear storage modulus (G'), which gives information about the fluid's elastic character and is related to the energy stored in the fluid during deformation, and the shear loss modulus (G''), which describes the fluid's viscous character and is related to the energy dissipated as heat during flow. The viscoelastic properties of the synovial fluid samples were assessed with a sweeping oscillatory frequency that included the physiological frequencies of knee movement, ranging from 0.01 Hz and 0.1 Hz (slower knee movements, such as occur while at rest and walking, respectively) to 3–4 Hz (more rapid knee movements, such as occur during running).

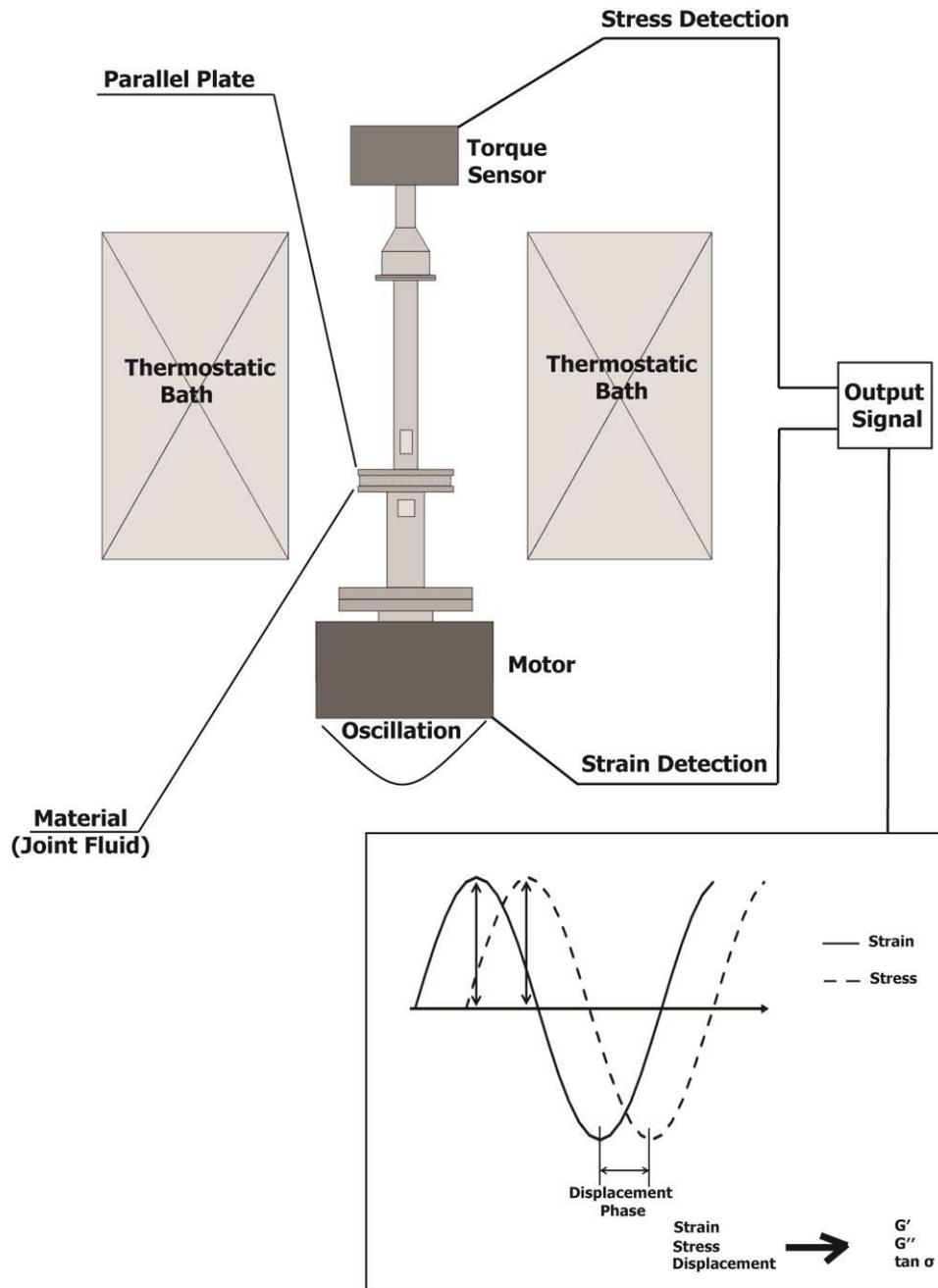


Figure 4. Illustration of the Dynamic Mechanical Analyzer (DMA) used in this study. G' = shear storage modulus (measuring the fluid's elastic character); G'' = shear loss modulus (measuring the fluid's viscous character); $\tan \delta$ = loss rate of energy of a mode of oscillation (measuring the phase displacement).

Statistical analysis

Data presentation: The data are presented as mean \pm standard deviation (SD). Data were considered independently with the assumption of a Gaussian distribution.

Statistical tests: The statistical analysis of all data was performed using one-way ANOVA followed by Tukey's post-hoc test with confidence intervals (CIs) of 95%. *P*-values less than 0.05 were considered significant. All analyses were performed using GraphPad Prism (GraphPad Software, Inc.).

Results

Perioperative conditions

All operations were performed smoothly without complications, such as joint sepsis or contractures. When the animals were killed, there was no significant variation in the body weights of the experimental groups, and the complete transection of all anterior cruciate ligaments was confirmed grossly. The joint fluid samples were generally clear or slightly blood tinged, with no gross signs of inflammation or infection; their volumes were in the range of 200–400 μL for samples from HA-injected knees and approximately 100 μL for samples from saline-injected knees.

Gross pathology

Using India ink and a previously described grading system³⁷, all knees were stained and graded at week 8 after ACLT. All stained knees showed some degree of degenerative changes, ranging from mild to severe, which were, in some cases, accompanied by joint effusion and synovitis. The femoral condyles exhibited more severe changes than the tibial plateaus, which tended to show mild changes (Fig. 2). All the samples from the sham group were Grade 1. For the OA groups, the NS and HA80 groups showed fairly extensive changes, mainly at the medial femoral condyle, whereas the HA600 group showed milder degeneration. The mildest changes (Grade 2: minimal fibrillation) were found in 10% of the NS group, 20% of the HA80 group, and 40% of the HA600 group, whereas the severest changes (Grade 4b: erosion ≥ 2 mm and < 5 mm) were found in 30% of the NS group and 20% of the HA80 and HA600 groups. No samples of grade 4c were detected (Fig. 5).

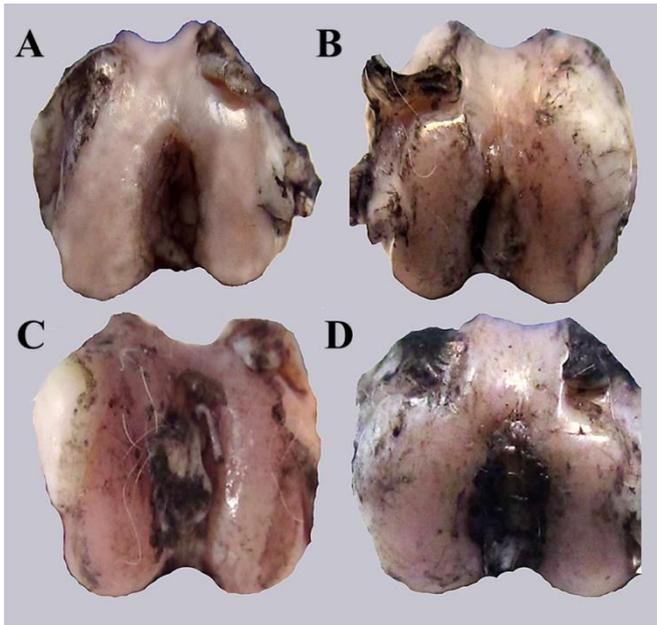


Figure 5. Macroscopic findings of femoral cartilage stained with India ink at 8 weeks after anterior cruciate ligament transection (ACLT). Sham (A); NS (B); HA80 (C); HA600 (D).

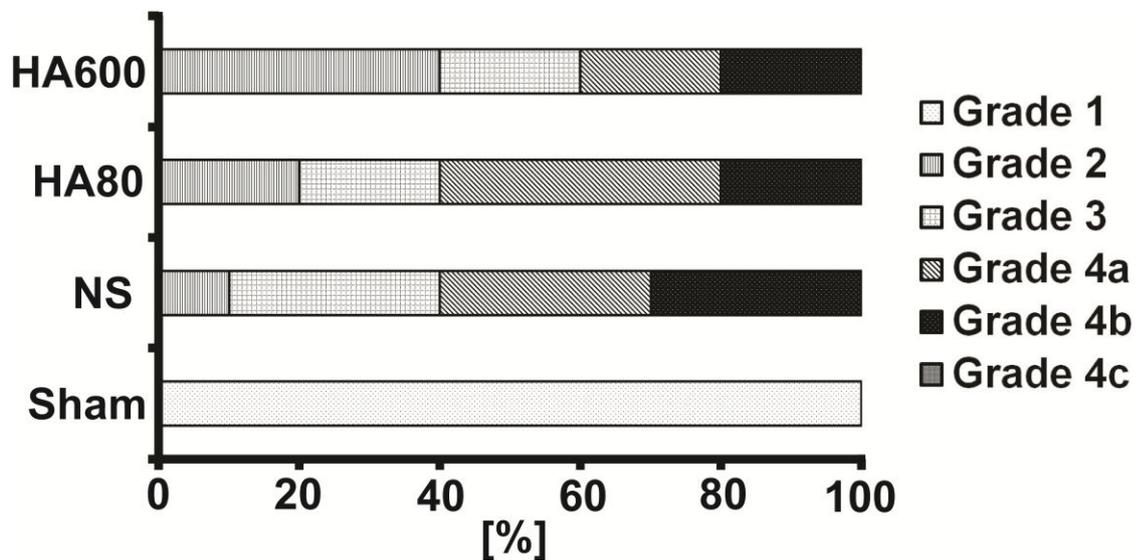


Figure 6. Macroscopic assessment of the medial femoral condyles from each group 8 weeks after surgery. Cartilage degeneration in the HA600 group was generally less severe than that in either the NS or HA80 group (n = 5 per group).

Histopathology

Microscopic examination of the articular cartilage showed varying degrees of degenerative change in all the knees from the three groups. The severest changes, such as the loss of the superficial layer, fibrillation/fissures, and the loss of Safranin-O staining, were observed in the NS and HA80 groups (Fig. 7A, 7B), whereas samples from the HA600 and the sham group exhibited less severe changes (Fig. 7C, 7G). In the HA80 group, there was a moderate reduction in the severity of cartilage degeneration, whereas in the HA600 group, there was a clear and marked reduction in the severity of the lesions. The loss of Safranin-O staining at the medial femoral condyle was also milder in the HA600 group than in the HA80 group. The overall scores for the HA600 group were significantly lower than those for the NS group ($P = 0.007$). The sham group showed significantly better overall scores compared with the NS ($P < 0.0001$), the HA80 ($P < 0.0001$), and the HA600 groups ($P = 0.0009$). The HA600 group also showed significantly better scores for the loss of the superficial layer and the loss of proteoglycans, whereas only the loss of the superficial layer was significantly better when the HA600 group was compared with the HA80 group ($P = 0.004$). The sham group exhibited better scores for most of the scoring system categories compared with the NS and HA80 groups (Table III). Although the overall scores for the HA600 group tended to be lower than those for the HA80 group, there was no statistically significant difference between the two groups. This was also true when the NS and HA80 groups were compared, and although the HA80 scores tended to be lower than those of the NS group, the differences were not statistically significant. The histological scores (8 = normal to 32 = the severest OA) for the medial femoral condyle are shown in Table III and figure 8.

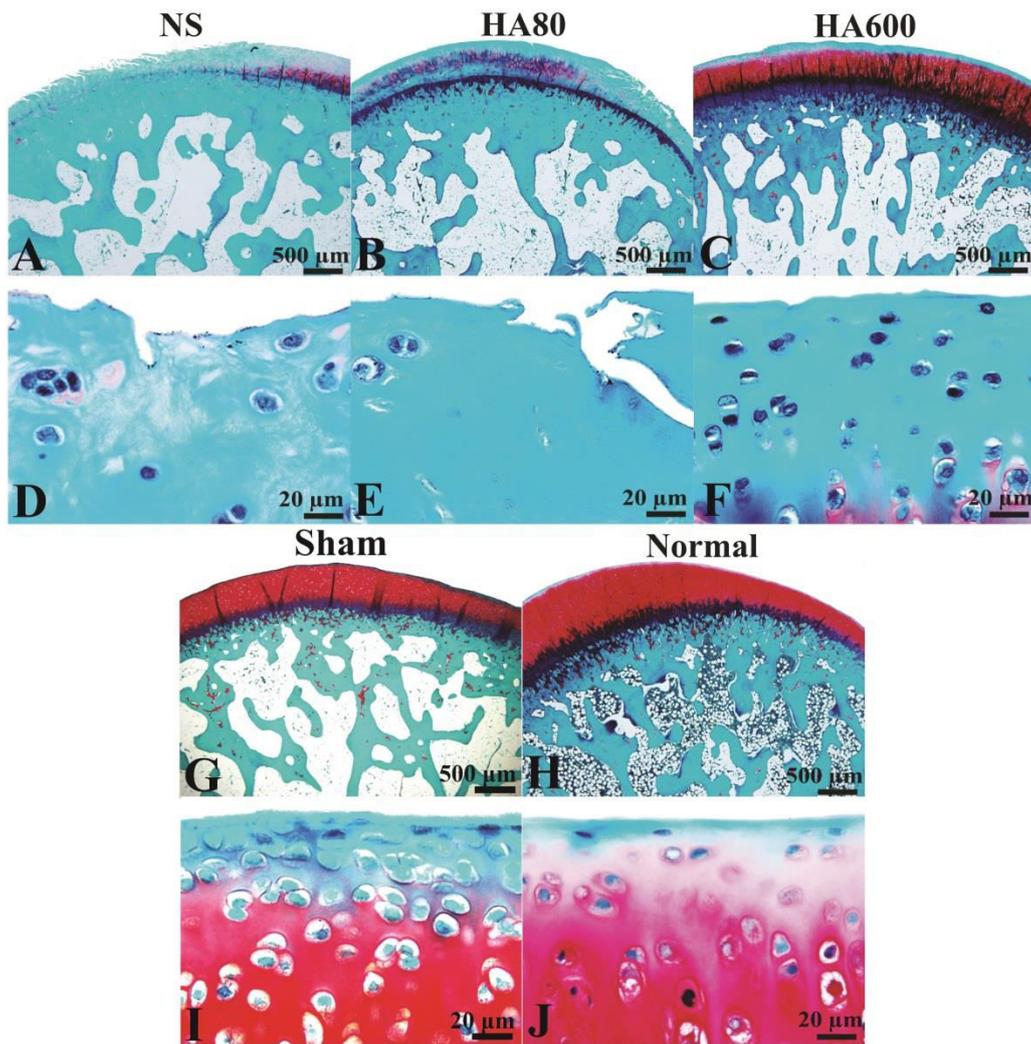


Figure 7. Histological findings (Safranin-O stain) of the medial femoral condyles from each group 8 weeks after surgery. NS (A, D); HA80 (B, E); HA600 (C, F); sham (G, I); normal knee that had not been operated on (H, J).

Table III. Mean histopathological scores for the medial femoral condyles in each group 8 weeks after anterior cruciate ligament transaction

Category	Sham (n = 5)	NS (n = 5)	HA80 (n = 5)	HA600 (n = 5)
A- Loss of superficial layer	1.13± 0.18*§	2.53 ± 0.38	2.6 ± 0.43	1.53 ± 0.56 ^{a,b}
B- Erosion of cartilage	1.2± 0.18*§	2.47 ± 0.45	2.4 ± 0.55	1.8 ± 0.61
C- Fibrillation and/or fissures	1.07± 0.15*§	2.4 ± 0.72	2.33 ± 0.62	2 ± 0.71
D- Loss of proteoglycans	1.2± 0.18*§¶	2.67 ± 0.33	2.27 ± 0.43	1.93 ± 0.36 ^c
E-Disorganization of chondrocytes	1.47± 0.18*§¶	3.13 ± 0.29	2.23 ± 0.43 ^e	2.47 ± 0.45 ^d
F- Loss of chondrocytes	1.07± 0.15*§	2.87 ± 0.61	2.47 ± 0.87	2.07 ± 0.28
G- Exposure of subchondral bone	1± 0.0 [§]	1.2 ± 0.18	1.8 ± 0.65	1.2 ± 0.45
H- Cluster formation	1.2± 0.18*§¶	2.86 ± 0.38	2.13 ± 0.56	2.33 ± 0.53
Sum	9.33± 0.24 ^{○#}	20.13±1.28	18.27 ±2.28	15.33±2.9 ^e

Mean ± SD.

*,§,¶P < 0.05 vs NS, HA80, and HA600, respectively; ○P < 0.0001 vs NS, and HA80; #P = 0.0009 vs HA600; aP = 0.012 vs NS; bP = 0.004 vs HA80; cP = 0.018 vs NS; dP = 0.04 vs NS; and eP = 0.007 vs NS.

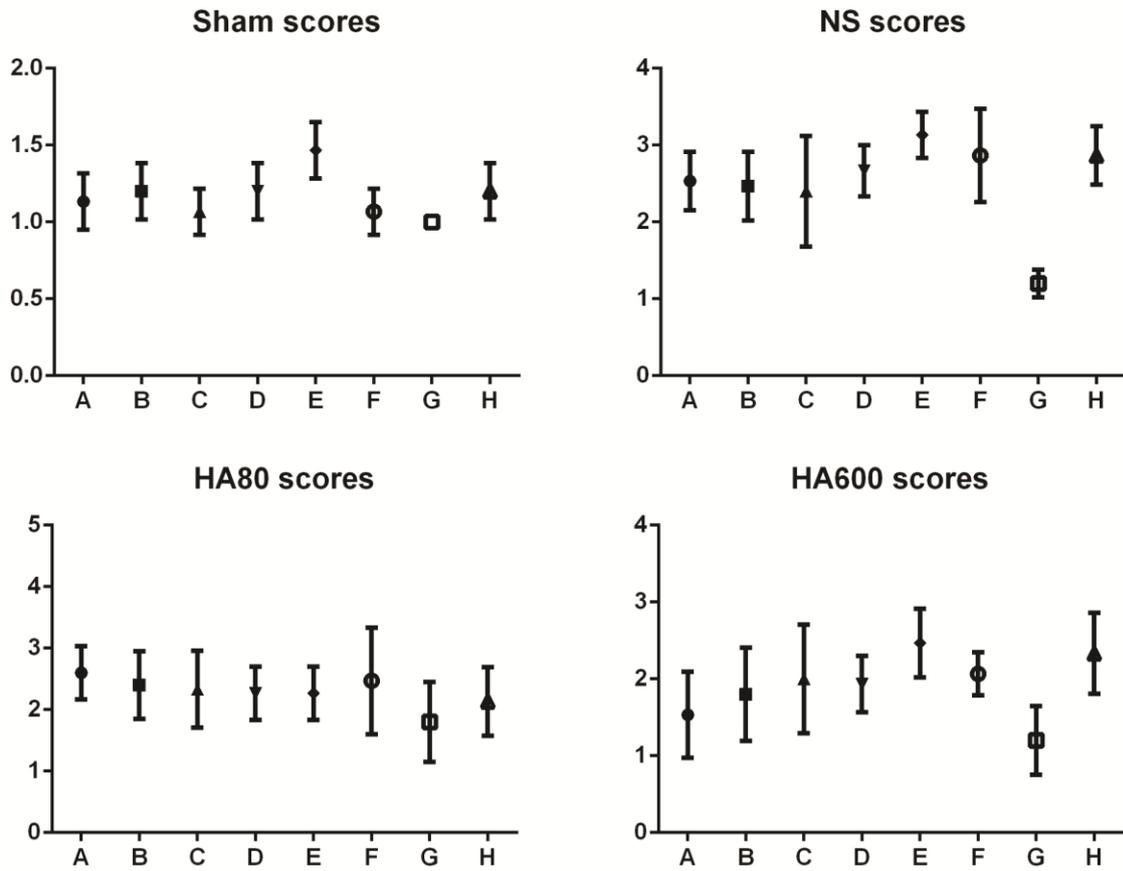


Figure 8. Detailed histological scores of each group; represented by Mean \pm SD for each assessment category.

Friction test

All knees were tested biomechanically to evaluate the friction coefficients. The friction coefficients were measured using the previously described system while the knees were under 60° flexion. The mean friction coefficient of the HA600 group was significantly lower than that of the NS group ($P = 0.002$), whereas there was no significant difference between the NS and HA80 groups or between the HA80 and HA600 groups, although the values found for the HA600 group tended to be lower than those for the HA80 group (Fig. 9).

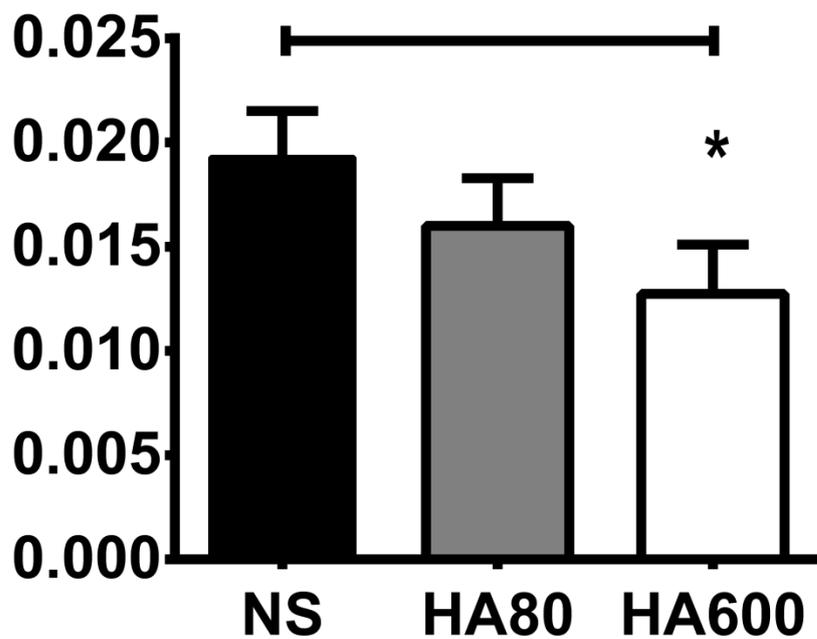


Figure 9. Mean values for the friction coefficients obtained with the pendulum friction test. (n = 5 per group). * $P = 0.002$ of HA600 vs the NS control. Mean \pm SD.

Dynamic mechanical analysis

Using the previously described apparatus, the rheological properties of the synovial fluid samples from the three groups (NS, HA80, and HA600) were recorded. Neither the G' nor the G'' measurements differed significantly between the three groups (Table IV).

Table IV. Viscoelasticity of normal joint fluid samples from each group compared with the viscoelasticity of the injected HA preparation measured at different frequencies (Hz)

	G' at Hz (Pa) (n = 5)				G'' at Hz (Pa) (n = 5)			
	0.01 Hz	0.1 Hz	1 Hz	4 Hz	0.01 Hz	0.1 Hz	1 Hz	4 Hz
NS	0.05± 0.03	0.1± 0.07	0.2± 0.13	0.26± 0.18	0.06± 0.04	0.11± 0.07	0.15± 0.08	0.19± 0.08
HA80	0.02± 0.02	0.05± 0.03	0.14± 0.07	0.23± 0.15	0.04± 0.03	0.1± 0.08	0.2 ± 0.17	0.24± 0.26
HA600	0.05± 0.01	0.1± 0.03	0.15± 0.06	0.24± 0.13	0.07± 0.03	0.14± 0.06	0.2 ± 0.09	0.25± 0.13

Means \pm SD (n = 5). NS = normal saline; HA = hyaluronic acid. G' = shear storage modulus (measuring the fluid's elastic character); G'' = shear loss modulus (measuring the fluid's viscous character); Hz = Hertz; Pa = Pascal

Discussion

I examined the chondroprotective effects of high-MW CL HA on knee OA induced with ACLT in rabbits. This OA model showed obvious degenerative changes in the articular cartilage 8 weeks after surgery, which resembled those observed in humans. The sham group did not show degenerative changes, thus excluding the possibility of spontaneous OA. Histological analysis showed that the intra-articular injection of HA inhibited OA progression significantly. Within the three treatment groups, less OA progression and better friction coefficients were seen in the HA600 group than in the HA80 and NS groups. These results indicate that intra-articular injections of HA600 have a chondroprotective effect on the articular cartilage.

A number of articles have investigated the effects of different MWs of HA^{38,41-44}. Kikuchi *et al.* demonstrated that the injection of low-MW HA (0.8×10^6 Da) did not significantly reduce OA progression compared with NS injection; other reports from Kawano *et al.* and Igarashi *et al.* showed similar results. These histopathological results are consistent with those reports.

On the other hand, data from Shimizu *et al.* showed that low-MW HA exhibited a cartilage-protective effect equal to that of high-MW CL HA⁴⁴. In our bilateral ACLT model, OA progression is expected to differ from that in their unilateral model, and the different number of injections, especially for low-MW HA, may affect the outcome. Recent literature reported a new highly cross-linked HA (Variofill®), and the results suggested a better pain relief effect compared with Synvisc®^{45,46}. However, I have no data on the analgesic effects of these materials. Future study is needed to investigate the effects of different types of high-MW CL HA.

In the histological analysis, the HA600 group had lower scores for the losses of the superficial layer, proteoglycans, and chondrocytes. Biomechanical testing showed that HA600 yielded significantly lower friction coefficients in the ACLT knees than did saline injections. Therefore, based on the friction test results, high-MW HA considered to act as a chondroprotective factor.

A previous article reported that the superficial zone of the articular cartilage does not have special properties that enhance its frictional response⁴⁷. In contrast, several investigators have shown that lubricin (a superficial zone protein, also called proteoglycan 4), which is present both in the superficial layer of the articular cartilage and in the synovial fluid, is very important in joint biomechanics and boundary lubrication^{48,49}. Another recent report also showed that supplementation with proteoglycan 4 can restore normal cartilage boundary lubrication to osteoarthritic synovial fluid⁵⁰. Synovial fluid from an individual with genetic deficiency of lubricin provides an opportunity to address the contribution of lubricin to synovial fluid's biophysical properties; the autosomal recessive human disease camptodactyly-arthropathy-coxa-vara-pericarditis (CACP) syndrome is caused by truncating mutations in the gene PRG4, leading to no lubricin expression^{51,52}. When comparing the synovial fluid from patients with osteoarthritis, rheumatoid arthritis, and CACP it was found that lubricin is the predominant component missing from the latter⁵³. Also a Lubricin knockout mice (Prg4^{-/-}) demonstrated increased joint friction and chondrocyte apoptosis in absence of inflammation⁵⁴. Although I did not investigate the lubricin in the cartilage immunohistochemically, my histological findings show clearly the lack of a superficial zone on the surface of the cartilage in all experimental groups, except the normal knee cartilage. The greatest histological difference between the three groups was in the uppermost layer of the cartilage. In the present study, I defined the uppermost ~100 μm as the superficial layer. I found that the cellular abundance and cellular shape in the uppermost layer in the HA600 group were similar to those in normal unoperated knees, whereas the superficial layer was almost acellular, with no proteoglycans, in the NS and HA80 groups. Seror *et al.* reported that the aggrecan-HA layer is a much better boundary lubricant than HA alone⁵⁵, and HA supplementation is also suggested to suppress proteoglycan loss⁵⁶. Of interest, a previous *in vitro* study demonstrated that changes in the MW of HA can affect synovial fluid's cartilage boundary lubricating ability in combination with the physiological levels of proteoglycan 4⁵⁷. A more recent study showed that high-MW HA can improve the cartilage integrity and might also stimulate cartilage repair by increasing collagen II and inhibiting interleukin-1 β -mediated matrix degradation by reducing matrix metalloproteinases⁵⁸. These data support my histological finding that HA600 preserved the superficial layer and improved the proteoglycan content.

Synovial joint lubrication involves two elements: fluid lubrication and boundary lubrication^{59,60}. The viscoelastic behavior of high-MW HA may affect the fluid lubrication in the knee joint. The elasticity and viscosity of Hylan G-F 20 are significantly greater than those of low-MW HA under *in vitro* conditions. However, in this study, measurements of the viscoelasticity of the joint fluid 1 week after the last injection showed no significant differences between the three experimental groups. I consider boundary lubrication to be the main component of the lubrication afforded by these test samples of HA600. The longitudinal rheological evaluation of the joint fluid was limited because the volume of the fluid was small in this rabbit OA model. Changes in the viscoelastic properties of HA and its MW after injection should be clarified in a future study.

This study had several limitations. First, although I am confident that the control injection of NS had little effect on joint lubrication, saline might slightly affect joint lubrication and the rheological characteristics of the synovial fluid. Further, although I repeated the *in vitro* rheological tests on joint fluids at 25 °C and 37 °C and found no significant differences (data not shown), I conducted no *in vivo* evaluations, which limits the generalizability of the findings. Second, I could not evaluate any changes in the MW of high-MW CL HA *in vivo* because the molecules are very large, making their exact measurement unfeasible. Third, I examined the viscoelasticity measurements at only one time point: 1 week after the third injection. I suspect that measurements should be made at earlier time points to clarify the evolution of differences in viscoelasticity produced by the different injection materials. Finally, I acknowledge that the small sample size and the young age, and thus skeletal immaturity, of the test animals limit the generalizability of the data.

Summary and Conclusion

Osteoarthritis represents a huge socioeconomic burden on the whole world with a very high prevalence rate. Although hyaluronic acid injection is one of the methods currently used for treating osteoarthritis patients as a non-operative measure, yet its effectiveness remains controversial.

This study has investigated two of the currently used hyaluronic acid preparations with different molecular weights and checked their chondroprotective effects in terms of histopathological findings, biomechanics and joint lubrication; using an experimental osteoarthritis model in anterior cruciate ligament transected knees of rabbits.

Findings showed that the intra-articular injection of high molecular weight cross linked hyaluronic acid is potentially chondroprotective, improving joint lubrication in osteoarthritic knees induced with anterior cruciate ligament transection. These attributes should contribute to the retardation of OA progression and consequently modify the pathological processes of osteoarthritis.

The clinical relevance of this study can be explained from the point that it used OA model which showed moderate arthritic changes in the operated knees, the injection materials used either the low MW HA or the high MW-CL HA are the same preparations currently available for patient use. The study showed clearly the chondroprotective effects of the high MW-CL HA and how it differ from the low MW HA and the normal saline control. This should be of help for the physician as it provides additional insights regarding the outcome of intra-articular injection of osteoarthritic knees. This evidence should be useful when selecting hyaluronic acid preparations for intra-articular injection procedures and for patient selection as well.

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