Evaluation of the Chondroprotective Effects of Glucosamine and Fish Collagen Peptide on a Rabbit ACLT Model Using Serum Biomarkers

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ABSTRACT. The aim of this study was to investigate the correlations of severity of osteoarthritis (OA) and serum biomarkers including keratan sulfate (KS), hyaluronic acid (HA) and chondroitin sulfate (CS) 846 epitope. We also investigated the effect of glucosamine and fish collagen peptide (FCP) on OA. OA was induced in 12 rabbits (12 weeks of age) by anterior cruciate ligament transection (ACLT). After the surgery, the rabbits were orally administered FCP (F group), glucosamine (G group) or FCP and glucosamine (FG group) for 4 weeks. The control group was provided water ad libitum (C group). Blood was collected before surgery (pre-ACLT) and before euthanasia (post-ACLT) for serum marker measurement. Biomarker levels were measured by using commercial kits. We evaluated OA severity both macroscopically and histologically. Macroscopic evaluation showed mildly eroded condylar surfaces in the C group. Histological findings were significantly different from the FG and other groups. There were no significant differences between each group at post-ACLT in terms of serum KS, HA and CS 846. Histological assessment and serum biomarker measurements performed at post-ACLT showed a significant correlation between HA concentration and OA severity. Variations in the CS 846 concentration at pre-ACLT and post-ACLT were significantly different from the FG and other groups. There were no significant differences between each group at post-ACLT in terms of OA severity. Glucosamine and FCP had chondroprotective effects in the ACLT model. Serum biomarker concentrations were significantly correlated with cartilage injury. Serum biomarker measurement would be useful for monitoring articular cartilage damage in the clinical setting.

KEYWORDS: biomarkers, fish collagen peptide, glucosamine, osteoarthritis.


Osteoarthritis (OA), the most common joint disease, is induced by cartilage loss that leads to joint destruction and severely impaired mobility. OA occurs in all mammals, including horses, dogs and humans. Degradation of the articular cartilage in the affected joint is the major feature of OA, which can be defined as a process of aberrant repair with gradual and progressive loss of articular cartilage through degradative mechanisms. Progressive deterioration of the articular cartilage leads to function loss and, ultimately, joint failure [8].

Cartilage is predominantly composed of collagen, with lesser amounts of proteoglycan (PG) and glycoprotein [7, 14]. Since adult cartilage contains neither a vascular nor a neural supply, chondrocytes primarily metabolize anaerobically, obtaining their nutrition via the synovial fluid, by means of diffusion [8].

There is currently no effective medical cure for OA. The only available treatments aim at reducing symptoms such as pain and inflammation, maintaining joint mobility and limiting function loss [10]. The main goals of ideal OA treatment include modifying symptoms, reducing pain and inflammation, modifying the structure, sparing the joint structure and preventing joint degradation to maintain articular function [10]. Undoubtedly, the discovery of biochemical markers for early OA detection will be helpful in the development of new pharmacological treatments aiming at arresting OA, before it becomes irreversible [4]. OA biomarkers should be specific to the diseased tissue and pathology, sensitive to changes in disease progression or therapeutic intervention, and predictive of disease outcome. However, an ideal multifunctional biomarker of OA has yet to be identified. It is unlikely that a single biomarker will ever meet all of the necessary criteria required for the complex diagnosis and observation of OA [8], so several biomarkers should be identified to enable more accurate OA diagnosis and evaluation.

Glucosamine, an amino monosaccharide, is synthesized from glucose, changing to glycosaminoglycan and contributing to maintenance of cartilage strength, flexibility and elasticity [15]. In several double-blind European studies in the early 1980s, investigators reported that oral glucosamine decreased pain, improved mobility and showed no side effects in human OA [3, 13, 22, 27].

Collagen is obtained by the enzymatic hydrolysis of collagenous tissue (bone, skin or scale). The main characteristic of collagen is its amino acid composition, which is identical to that of type II collagen, thus providing high levels of glycine and proline, 2 amino acids essential for cartilage stability and regeneration [30]. For some time, collagen has been reported to have beneficial biological functions that justify its use in food supplements and pharmaceutical preparations. Clinical investigations suggest that use of a collagen dietary...
supplement can improve joint pain and mobility [1, 2]. The aim of this study was to investigate the correlation between OA severity and concentration of serum biomarkers such as keratan sulfate (KS), hyaluronic acid (HA) and chondroitin sulfate (CS) 846 epitope. Therefore, we researched the effect of glucosamine and fish collagen peptide (FCP) on ACLT in rabbits. We then assessed the correlations between serum biomarkers and histological findings.

MATERIALS AND METHODS

Materials: Animals.Twelve clinically healthy rabbits (female Japanese albino; average age, 12 weeks) with a body weight of 2.5–3.0 kg were used. The animals were used in the experiment after 1-week acclimatization to the laboratory environment.

FCP. Collagen was extracted from skins of Gadiformes species (Yaizu Suisankagaku Industry Co., Ltd., Shizuoka, Japan) and degraded by proteinase to obtain peptides of various sizes with lower molecular weights. The mean molecular weight of the prepared FCP was 3,000. The major amino acids constituting FCP include glycine (24.6% of the dry matter), glutamic acid (10.8%), proline (10.6%) and alanine (9.5%).

Glucosamine. Chitosan obtained from shrimp shells was transformed into glucosamine via HCl treatment (Yaizu Suisankagaku Industry Co., Ltd.). The glucosamine we used was of >95% purity.

Methods: All experimental procedures were approved by the animal care and use committees of Tottori University and conducted in accordance with The American Physiological Society’s guiding principles in the care and use of animals. The experimental rabbits (n=12) were divided into 4 groups, namely the control group (C group), the group receiving FCP (F group), the group receiving glucosamine (G group) and the group receiving FCP and glucosamine (FG group). Three rabbits were used in each group. First administered FCP and glucosamine after surgery and continued doing so for 4 weeks.

Oral administration of glucosamine and FCP: The C group had free access to tap water. The rabbits in the other groups were also allowed to drink tap water, after we ensured that the daily dosage of each agent was ingested. For each animal in the F group, 1.0 g of powdered FCP/day was dissolved into half the average daily quantity of drinking water, which was orally administered. The G group received 1.0 g of glucosamine, while the FG group received 1.0 g of FCP and 1.0 g of glucosamine daily.

ACLT surgery: An analgesic (xylazine hydrochloride, 10 mg/kg) was administered as premedication. After sedation, induction anesthesia was performed in a box by inhalation of a mixture of 5% isoflurane in oxygen. Anesthesia was maintained by inhalation of a mixture of 3% isoflurane in oxygen using a mask. A limb was clipped and prepared for surgery in a standard aseptic manner. A medial parapatellar incision was made through the skin, and an arthrotomy was performed. The patella was dislocated laterally, and the knee was placed in full flexion. The ACL was visualized and transected with a no. 15 blade. The joint was then irrigated with sterile saline and closed. The joint capsule with subcutaneous tissue was closed using interrupted absorbable sutures (3-0 Maxon; Johnson & Johnson, New Brunswick, NJ, U.S.A.), and the skin incision was closed with interrupted sutures (Wire Spool; Kirikan Ltd., Tokyo, Japan). During the week following the operation, 10 mg/kg of oxytetracycline (Terramycin; Pfizer, Tokyo, Japan) was administered subcutaneously twice a day to prevent infection.

Post-mortem examination: At 4 weeks after the surgery, the rabbits were euthanized by an intravenously administered overdose (80 mg/kg) of pentobarbital (Nembutal; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). The knee joints were opened, and the injured cartilage at operated site was macroscopically observed. Evaluation of injured sites by histological findings: Histological assessment of the cartilage was performed on the rabbit femurs in each group. The femoral articular surfaces were fixed in 10% neutral buffered formalin. Tissue blocks were decalcified with 14% EDTA in 10 mM phosphate buffer (pH 7.4), dehydrated through graded alcohols, rinsed in toluol and embedded in paraffin. Sections (6 µm) were cut at standard central sites in the medial femoral condyle (MFC) and lateral femoral condyle (LFC). The sections were stained Safranin O-Fast green or hematoxylin-eosin-saffron. Immunohistochemical staining was performed using antitype II collagen mouse monoclonal antibody. The sections from each site and their averages were evaluated by criteria using a modified Mankin grading system as shown in Table 1. Furthermore, we also assessed type II collagen loss. The sites of histological assessment were divided into 2 parts, MFC and LFC, each of which was evaluated and averaged.

Measurements for serum biomarkers: Blood was collected before surgery (pre-ACLT) and before euthanasia (post-ACLT). The sera were separated and immediately stored on ice. The collected sera were frozen at −80°C for long-term storage before estimations of CS 846 epitope, HA and KS were made. Each assay was performed using an Aggrecan Chondroitin Sulfate 846 Epitope ELISA kit (IBEX Technologies, Inc., Montreal, Canada), QnE Hyaluronic Acid (HA) ELISA kit (Biotec Trading Partners, Inc., Encinitas, CA, U.S.A.) and a Keratan Sulfate ELISA kit (Seikagaku Biobusiness, Tokyo, Japan).

Statistical analyses: All analyses were planned to be performed at a 5% significance level. For histological assessment, differences between animal groups were analyzed with one-way ANOVA and multiple comparisons (Scheffe’s F test). Post-ACLT biomarker levels between each group were analyzed with the Kruskal-Wallis test. Post-ACLT as-
associations between histological assessments and biomarker levels were examined using Pearson’s correlation coefficient test, if normality of the groups was confirmed. Spearman’s rank-correlation coefficients were calculated, if normality of the groups was not identified. Associations between histological assessments and variations in biomarker levels (post-ACLT value − pre-ACLT value) were also identified in the same manner.

RESULTS

**Macroscopic and histological findings and assessment scores:** Macroscopic findings at 4 weeks post surgery are shown in Fig. 1. In the C group, the condyle surfaces had mild erosion (Fig. 1A). In the histological findings of the C group at 4 weeks post surgery, the femoral condyle surfaces were covered with fibrous tissue instead of cartilaginous tissue. Decreasing levels of chondrocytes, PG and type II collagen were obvious compared with the normal femoral condyle (Fig. 2A, 2a, 2B and 2b). The decreasing in PG, type II collagen and chondrocyte levels in the F, G and FG groups (Fig. 2C, 2c, 2D and 2d) were mild. These histological findings were similar in 3 zones.

The total histological assessment scores of all sites (MFC, LFC and average) are shown in Table 2. The average score was estimated using the following equation: (MFC score + LFC score)/2. Decreased Safranin O staining and type II collagen immunostaining after administration of FCP and glucosamine tended to occur in the F, G and FG groups as compared with the C group. These results indicate that the administration of FCP and glucosamine prevented ACLT-induced articular cartilage degradation.

The FG group had significantly lower mean total global LFC and average compartment scores compared with the C group (P<0.01 and P<0.05, respectively). Differences between the FG group and F and G group were also significant (P<0.05, both) in the LFC compartment.

**Serum biomarkers:** The measurement values are shown in Fig. 3. There were no significant differences between the pre-ACLT and post-ACLT values of serum KS, HA or CS 846. In terms of the post-ACLT values, there were no significant differences in serum KS, HA and CS 846 between groups.

**Correlation between histological assessment and serum biomarkers:** Correlations between histological assessment and serum biomarkers were evaluated using the post-ACLT...
concentrations and variations (post-ACLT value − pre-ACLT value) of each biomarker concentration (KS, Fig. 4; HA, Fig. 5; CS 846, Fig. 6; ♦, C group; ■, F group; ▲, G group; ×, FG group).

The correlation between histological assessment and post-ACLT HA values was significant for MFC and average scores ($P<0.05$, both, Fig. 5A).

Variations between pre-ACLT and post-ACLT were significantly correlated with all site scores (MFC, LFC and average) for CS 846 ($P<0.001$, 0.001 and 0.001, respectively, Fig. 6B).

**DISCUSSION**

In the present study, we investigated cartilaginous tissue degradation by measuring serum KS, HA and CS 846 levels at 4 weeks using an ACLT surgical model and evaluated the chondroprotective effect of FCP and glucosamine administration. To our knowledge, this study is the first to evaluate the chondroprotective effect of FCP and glucosamine on an ACLT model, as well as the correlation between histological findings and serum biomarker levels.

The results of the histological assessment showed that the FG group had experienced a chondroprotective effect, as evidenced by the amount of PG, type II collagen and chondrocytes in the cartilaginous tissue compared with the C group.

**Fig. 1.** Macroscopic findings at 4 weeks post surgery. A, control (C) group; B, fish collagen peptide (F) group; C, glucosamine (G) group; D, fish collagen peptide + glucosamine (FG) group. In the C group, the surface of the condyle has mild cartilage erosions (arrows). However, no erosion was seen in the other groups (F, G, FG).

**Fig. 2.** Histological findings of the lateral femoral condyle in the C and FG groups at 4 weeks post surgery. S, superficial zone; M, middle zone; D, deep zone; SCB, subchondral bone. A: Low-magnification findings; 3 zones (S, M, D) and SCB. The cleft extends into the middle zone. There is a loss of red Safranin O staining in all 3 zones. a: High-magnification findings of the inset in A, in which there is a marked decrease in chondrocytes. Red Safranin O staining around the cells is not seen. B: Low magnification of type II collagen immunostaining, in which the surface is covered by fibrous instead of cartilaginous tissue. Loss of staining in all 3 zones is evident. b: High magnification of the inset in B. Staining of type II collagen is not seen. C: The surface shows only irregularities at lower magnification. The A and M zones show the loss of Safranin O staining, but staining is still seen in the D zone. c: High magnification of the inset in C. There are chondrocytes and red staining around the cells in the D zone. D: Low magnification of type II collagen immunostaining, in which the surface is covered by fibrous instead of cartilaginous tissue. Loss of staining in the S and M zones is shown but staining remains in the D zone. d: High magnification of the inset in D showing staining of type II collagen around the cells in the D zone.
These statistical differences were also found between the FG and F and G group. This might suggest that administration both FCP and glucosamine results in a synergistic chondroprotective effect.

There were no significant differences between the groups in terms of the post-ACLT values of serum KS, HA and CS epitope. This might suggest that administration of FCP, glucosamine or both results in no significant difference between groups.

Many researchers have attempted to detect the metabolic products of articular cartilage components in joint fluid or blood and thereby identify an OA marker. As reported by Okumura et al., early OA articular cartilage destruction begins with a loss of GAGs from the articular cartilage surface, followed by collagenolysis. Thus, the first event in OA or articular cartilage damage is the release of GAGs, which play an important role in maintaining articular function. Consequently, early markers of articular cartilage damage or OA changes might be found among GAG metabolic products. For this reason, we decided to research KS, HA and CS epitope as biomarkers.

KS is a component of proteoglycans found in the articular cartilage, intervertebral disc and corneas. Because corneas are relatively small tissues, serum KS is mainly originates from articular cartilage and intervertebral discs. Thus, the serum concentration of KS is not only a marker of knee articular cartilage, but is considered to reflect the normal metabolism of cartilage. Wakitani et al. suggested that serum KS might indicate the release of cartilaginous GAG in the early stage after injury despite moderate cartilaginous damage and might be used in a screening and monitoring test. Our result did not show that the post-ACLT serum KS values were significantly different between groups or that there was significant correlation with the histological assessment score. This might be the result of our experimental period; 4 weeks is too short to utilize serum KS as a biomarker for the early stage of OA. Therefore, investigation of KS as a biomarker for the early stage of OA requires a longer period.

<table>
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<th>Group</th>
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<th>Safranin O-Fast green staining</th>
<th>Type II collagen immunostaining</th>
<th>Chondrocyte loss</th>
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<td>1.50 ± 0.50</td>
<td>12.83 ± 1.04 **</td>
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Values are presented as means ± SD. (n=3). * The global score in the FG group at LFC is significant lower than in the other groups (P<0.05, FG group vs the F and G groups; P<0.01, FG group vs C group). ** The average global score in the FG group is significantly lower than in the C group (P<0.05).

Fig. 3. Pre- and post-anterior cruciate ligament transection (ACLT) serum biomarker concentrations (A, keratan sulfate [KS]; B, hyaluronic acid [HA]; C, chondroitin sulfate 846 epitope [CS 846]). Values are presented as means ± SD. (n=3). “Pre” is the average pre-ACLT value in all groups. The other bars indicate the post-ACLT levels of each group.
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HA is a constituent of the synovium and cartilage and is thought to contribute to the lubricating mechanisms of synovial fluid. Degradation and turnover of the extracellular matrix results in the release of HA. Its presence in serum can be caused by conditions other than arthritis. Nevertheless, increased serum levels of hyaluronic acid have been reported in patients with OA [6]. There is a report that showed that measurement of serum HA levels may be useful in assessing knee OA activity and determining predictive factors [25]. It was also reported that plasma HA levels in patients with OA directly correlated with the functional capacity and estimated burden of diseased cartilage [9]. Our results showed that the post-ACLT HA values were inversely correlated with histological cartilage damage. This discrepancy might be because serum HA levels are not elevated in the early stages of OA, such as at 4 weeks. Administration of both FCP and glucosamine might enhance the production of HA in synovial tissues [18, 27]. This might reflect the serum HA value regardless of OA severity.

CS is a GAG that is covalently attached to specific proteins to form proteoglycans, which are abundant within the extracellular matrix. The level of aggrecan synthesis can be evaluated by using antibodies against epitope 846, which is located on the chondroitin sulfate chains [24]. It was recently proposed that the presence of the CS 846 epitope may serve as a marker of newly synthesized aggrecan molecules in the matrix and that its appearance in joint fluid and serum could reflect the rate of aggrecan synthesis [23]. Conversely, ratios of 846 epitope to aggrecan were high in the more advanced stages of OA, suggesting that both synthesis and degradation of aggrecan were upregulated [11]. The correlation between

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**Fig. 4. Correlation between histological score and serum KS concentrations.** Scatter diagrams showing the correlation between the histological score and KS value at post-ACLT (A) and variation (B) at each site (MFC, LFC, and average). The y-axis indicates the post-ACLT values (A) and changes in values between pre- and post-ACLT (B). The x-axis shows the histological score of each site. The slope in the scatter plot represents the regression line.
EVALUATION IN AN ACLT MODEL USING BIOMARKERS

Histological cartilage damage and changes in CS 846 concentration indicates the aggrecan synthesis is enhanced in more severely damaged cartilage.

Collagen has been reported to have beneficial biological functions. The presence of collagen hydrolysates indicates a stimulatory effect of degraded collagen on type II collagen biosynthesis of chondrocytes in vitro and suggests a possible feedback mechanism for the regulation of collagen turnover in cartilage tissue [18]. In the STR/ort mouse model of spontaneous OA, oral administration of collagen hydrolysate could be of potential interest as a disease-modifying agent for the prevention of degenerative joint disease [19].

Pro-Hyp has been identified in the blood after oral intake of collagen hydrolysate [20]. Nakatani et al. demonstrated the chondroprotective effect of Pro-Hyp under pathological conditions in mouse in vivo and in vitro models [16]. Moreover, it has been suggested that food-derived Hyp-containing peptides can affect the PGs and morphological changes associated with OA cartilage, which might be mediated by stimulation of HA production in the synovium [19].

Glucosamine is an important precursor of glycoprotein and GAG synthesis. When glucosamine was first proposed as a treatment for OA, it was thought to augment the endogenous production of glucosamine and to enhance the synthesis of PG, which is lost early in OA [28]. Oral glucosamine has the effect of restoring GAG in damaged cartilage, but its mechanisms of action are unknown [17]. On the other hand, glucosamine was reported to have an effect on type II collagen synthesis [15]. In the rat OA model, glucosamine exerts a chondroprotective action by maintaining PG, inhibiting type II collagen degradation and enhancing type II collagen synthesis in the articular cartilage [15]. In addition, glucosamine has a positive effect on subchondral bone turnover, structure, and mineralization in the early stages of OA.

Fig. 5. Correlation between histological score and serum HA concentrations. The scatter diagrams show the correlation between the histological score and HA value at post-ACLT (A) and variation (B) at each site (MFC, LFC and average). The y-axis indicates the values at post-ACLT (A) and change in values between pre-ACLT and post-ACLT (B). The x-axis shows the histological score of each site. The slope in the scatter plot represents the regression line.
experimental OA [31]. It was reported that exogenous glucosamine can increase HA production of the synovial tissue [27]. This might also be useful for preventing OA development or progression.

From our present results, oral administration of FCP and/or glucosamine effectively controlled cartilage degradation in an ACLT model. Estimation of various biomarkers for arthritis will be useful for assessing the progression of cartilaginous degradation; in this present study, the detected levels of HA and CS 846 correlated with the histological findings, suggesting that estimation of HA and CS 846 might be useful for monitoring OA progression. However, there were no significant differences between the control and treatment group concentrations. These results indicate that there are individual differences for each biomarker; therefore, a longer-term experiment should be conducted to evaluate the significance of the biomarkers in the ACLT model. Our results indicate the possibility that the measured concentrations of biomarkers can be used in addition to histological findings to evaluate cartilage injury.

REFERENCES


