NOTE *Surgery*

Correlation of Plasma Amino Acid Concentrations and Chondroprotective Effects of Glucosamine and Fish Collagen Peptide on the Development of Osteoarthritis

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ABSTRACT. We investigated the correlation of plasma amino acid concentrations and the effects of glucosamine and fish collagen peptide (FCP) on osteoarthritis (OA). OA was induced according to the rabbit anterior cruciate ligament transection (ACLT) model. After surgery, the rabbits were orally administered FCP (F group), glucosamine (G group) or both (FG group) for 4 weeks. The control group (C group) was not administered anything. Blood was collected before surgery (pre-ACLT) and before euthanasia (post-ACLT). Changes in the alanine, threonine and methionine concentrations were significant between pre- and post-ACLT. The correlation between the histological assessment and arginine concentration post-ACLT was significant. These findings indicate that measurement of plasma amino acids is useful for evaluation of the efficacy of intervention for OA.

KEY WORDS: ACLT, amino acid, fish collagen peptide, glucosamine, osteoarthritis.

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Osteoarthritis (OA) is a common, debilitating joint disease that affects all mammals, the early stage of which is difficult to diagnose. Joint structure and function are typically altered substantially before symptoms cause patients to seek medical care; that is, the osteoarthritic process begins long before OA presents as a clinical disease [16]. This delays treatment that may help prevent further cartilage destruction and joint failure. Therefore, the discovery of biomarkers for early OA detection would be helpful in the development of new pharmacological treatments aimed at arresting OA before it becomes irreversible [9]. For many years, various techniques have been used to experimentally induce OA in animals. The rabbit anterior cruciate ligament transection (ACLT) model is being used increasingly in OA studies. We evaluated OA severity by examining the histological changes produced by the degradation of proteoglycan and type II collagen in the ACLT model.

Nutraceuticals are candidates for long-term prevention of chronic disease, such as OA [12]. Furthermore, they have gained increased recognition because they help maintain bone and joint health. Glucosamine and collagen hydrolysate are 2 such nutraceuticals, and many researchers have insisted on their effectiveness. We hypothesized that these supplemental chondroprotective mechanisms are related to plasma amino acids. Therefore, we investigated whether plasma amino acids are correlated with the development and severity of OA by comparing plasma amino acid concentrations between before ACLT and before euthanasia.

Animal model: Twelve clinically healthy rabbits (Japanese albino, females; average age, 12 weeks) with a body weight of 2.5–3.0 kg were used in the experiment after they were acclimatized to the laboratory environment for 1 week. Collagen was extracted from the skins of fish of the *Gadiformes* species and degraded by proteinase to obtain various sizes of peptides with lowered molecular weights (Yaizu Suisankagaku Industry, Ltd., Shizuoka, Japan). The mean molecular weight of the prepared FCP was 3,000 Da. The major amino acids constituting the FCP were glycine (24.6% of dry matter), glutamic acid (10.8%), proline (10.6%) and alanine (9.5%). Chitosan obtained from shrimp shells was transformed into glucosamine through HCl treatment (Yaizu Suisankagaku Industry, Ltd.). The glucosamine we used was >95% purity.

All experimental procedures were approved by the animal care and use committee of Tottori University, and were conducted in accordance with the "Guiding Principles in the Care and Use of Animals" of the American Physiological Society. The experimental rabbits (n=12) were divided into 4 groups, namely, the control (C group), a group receiving FCP (F group), a group receiving glucosamine (G group) and a group receiving both FCP and glucosamine (FG group). Three rabbits were included in each group. FCP and glucosamine were administered after surgery and continued for 4 weeks. The control group had free access to tap water. The rabbits in the other groups were also allowed free access to tap water after they had ingested the daily dosage of each agent. For the F group, 1.0 g of powered FCP/day was dissolved into half the average daily water requirements for each rabbit and orally administered. The G group received 1.0 g of glucosamine, and the FG group received 1.0 g of FCP and 1.0 g of glucosamine daily, as in the F group. An an-

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algesic (xylazine hydrochloride, 10 mg/kg) was administered as a premedication. After sedation, anesthesia was induced in an induction box through inhalation of 5% isoflurane in oxygen. Anesthesia was maintained using 3% isoflurane in oxygen delivered through a mask. The limb was clipped and prepared for surgery in a standard aseptic manner. A medial arthrotomy was performed on the femoropatellar joint to permit transection of the ACL. The joint capsule with the subcutaneous tissue was closed by interrupted sutures using absorbable suture material (3–0 Maxon, Johnson & Johnson, New Brunswick, NJ, U.S.A.), and skin incision was closed in the same manner with a stainless steel suture (Wire Spool, Kirikan Ltd., Tokyo, Japan). During the week following the operation, 10 mg/kg of oxytetracycline (Terramycin, Pfizer, Tokyo, Japan) was subcutaneously administered twice a day to prevent infection. Four weeks after 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 at the surgical site, and the injured cartilages were macroscopically observed.

Evaluation of histopathological change: Histologic assessment was performed on the femur in the rabbits of each group. The femoral articular surfaces were fixed in 10% neutral buffered formalin. Tissue blocks were decalcified with 14% EDTA in 10 mM of phosphate buffer (pH 7.4), dehydrated through graded series of alcohols, rinsed in toluol and embedded in paraffin. Six-micrometer sections were cut at a standard site centrally in the medial femoral condyle (MFC) and lateral femoral condyle (LFC). These sections were stained with Safranin O-fast green and hematoxylin-eosin-saffron. Immunohistochemical staining was also performed with anti-type II collagen mouse monoclonal antibody. The sections from each site and their average histological assessment were evaluated according to the modified Mankin grading system [22]. Furthermore, the loss of type II collagen was evaluated in the same manner as the loss of proteoglycan. We evaluated the difference between each group by averaging the MFC and LFC histological assessments. The average score was calculated as follows: (MFC score + LFC score)/2.

Analysis of plasma amino acids: Blood was collected before surgery (pre-ACLT) and before euthanasia (post-ACLT) by using heparin as anti-coagulant. The blood was centrifuged at 3,000 rpm for 10 min, and the plasma was then separated promptly and frozen at −80°C until the measurement of plasma amino acid concentrations. Plasma samples were mixed with equal volumes of 3% (w/w) sulfosalicylic acid, and left to stand at 4°C for 1 hr. Samples were the centrifuged (4°C, 15 min, 1,500 rpm), and the precipitated protein was removed. The amino acid concentrations were measured by an automatic amino acid analyzer (JLC-500/V2, AminoTac; JEOL, Tokyo, Japan). The amino acids measured were threonine (Thr), valine (Val), methionine (Met), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), lysine (Lys), histidine (His), tryptophan (Trp), aspartate (Asp), serine (Ser), arginine (Arg), glutamate (Glu), glutamine (Gln), glycine (Gly), alanine (Ala), tyrosine (Tyr), proline (Pro), citrulline (Cit), ornithine (Orn) and hydroxyproline (Hyp).

Statistical analysis: All analyses were carried out at a 5% significance level. For histological assessment, differences between the animal groups were analyzed with one-way ANOVA and multiple comparisons (Scheffe's F test). Post-ACLT associations between histological assessments and biomarker levels were determined using Pearson's correlation coefficient test if normality of the groups was confirmed. Spearman's rank correlation coefficient was used if normality of the groups was not identified. Associations between histological assessments and changes in biomarker levels (post-ACLT and pre-ACLT values) were also determined in the same manner.

Histopathological changes: At 4 weeks after the surgery, mild erosion was observed on the condyle surfaces in the C group; however, no difference was recognized macroscopically between groups. Histological findings of the C and FG groups for type II collagen immunostaining are shown in Fig. 1. The histological findings of the C group at 4 weeks after the surgery showed that the surface of the femoral condyle was covered tissue instead of cartilaginous tissue. Decreasing levels of chondrocytes, PG and type II collagen were obvious compared with the normal femoral condyle. These histological findings were almost the same in 3 zones. The total average histological assessment scores are shown in Fig. 2. A tendency for a decreased loss of Safranin O staining and type II collagen immunostaining was observed in the F and G groups as compared with the C group. The results indicate that the administration of FCP and glucosamine prevented articular cartilage degradation by ACLT. A significantly lower mean value of the total average global score was obtained in each compartment for the FG group compared with the C group (P=0.049).

Changes in the plasma amino acid levels: Significant changes in the amino acid levels, in particular for Ala, Thr and Met, between pre- and post-ACLT or changes in the plasma amino acid concentrations by ACLT-induced OA, were observed in all rabbits (Table 1). The correlation between the histological assessment and the concentration changes in each plasma amino acid was evaluated post-ACLT. Each average score was used as a histological assessment score. Significant negative correlation was observed for Arg (P<0.05) as well as for Glu, Hyp, Cit and Orn (0.05<P<0.10; Fig. 3). Significantly negative correlations between histological assessment scores and the plasma concentrations of these amino acids were observed post-ACLT.

In our study, we showed the presence of a negative correlation between OA development and the changes in plasma amino acid concentrations. Our results indicate that the chondroprotective effects were attributed to the administration of FCP and glucosamine. We found that the concentrations of certain amino acids changed during the development of OA were negatively correlated with the severity of OA. From the results of the histological assessment score, the FG group, compared with the C group, displayed significant chondroprotective effects on proteoglycan (PG) and type II collagen levels as well as the number of chondrocytes in the cartilaginous tissue. In the ACLT model, full-thickness

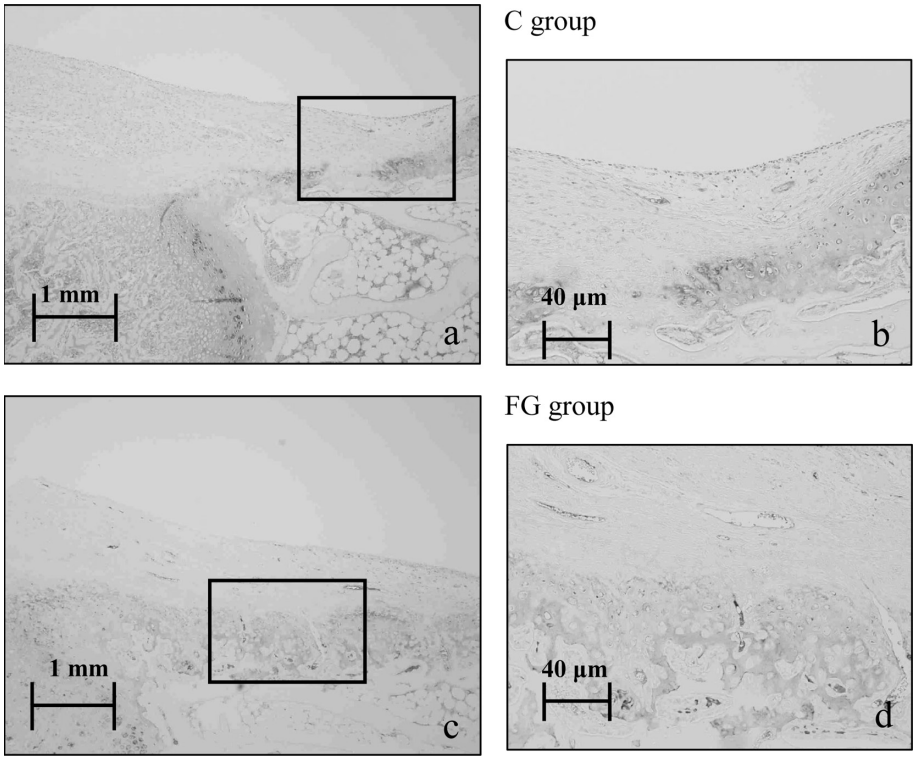

Fig. 1. Histologic findings of the lateral femoral condyle in the C and FG groups in type II collagen immunostaining at 4 weeks after surgery. a): At lower magnification, the surface is covered by fibrous instead of cartilaginous tissue. Collagen staining is only shown in the deep zone. b): Higher magnification of the inset in a). Staining of type II collagen is only seen in the deep zone. c): At lower magnification, the surface is covered by fibrous instead of cartilaginous tissue. The staining remains in the deep zone. d): Higher magnification of the inset in c) showing staining of type II collagen around the cells in the deep zone.

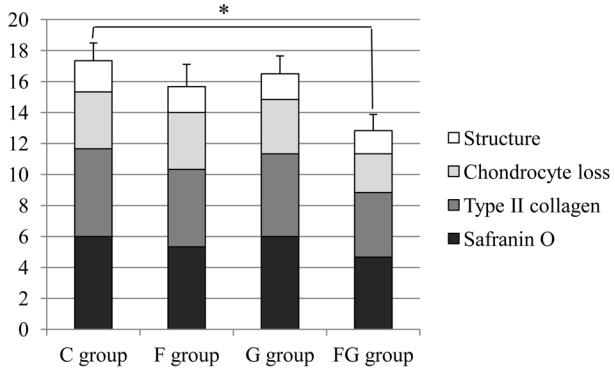

Fig. 2. Histological assessment score averages. Values are presented as means ± SD (n=3). *P<0.05.

Table 1. Significant differences in the plasma amino acid concentrations between pre- and post-ACLT in all rabbits (μg/ml)

	Pre-ACLT	Post-ACLT
Ala	48.01 ± 13.93	35.56 ± 7.13
Thr	19.38 ± 6.79	24.37 ± 4.02
Met	4.69 ± 1.94	6.68 ± 1.15

These amino acids showed significant differences between pre- and post-ACLT (P<0.05).

ulceration was noted after 4 weeks [26], and therefore, our experimental OA represented the early stages of the disease. The effects of glucosamine on OA are shown by inhibiting the degradation and stimulating the synthesis of proteoglycan [10, 11]. In addition, glucosamine is excepted to have anti-inflammatory action [13]. Collagens reportedly stimulate type II collagen and proteoglycan synthesis, as well as aggrecan expression by chondrocytes [19]. Collagens have also been shown to prevent chondrocyte differentiation into mineralized chondrocytes [17]. The detailed mechanisms indicating the effects of both glucosamine and collagen are not reported. However, the effects of both are similar in some respects. In addition, only the FG group showed a significant difference compared with the C group in the histological findings. Therefore, we evaluated if administration of both collagen and glucosamine has a synergetic effect. There are some reports indicating that collagen and glucosamine are useful for OA patients. In several European double-

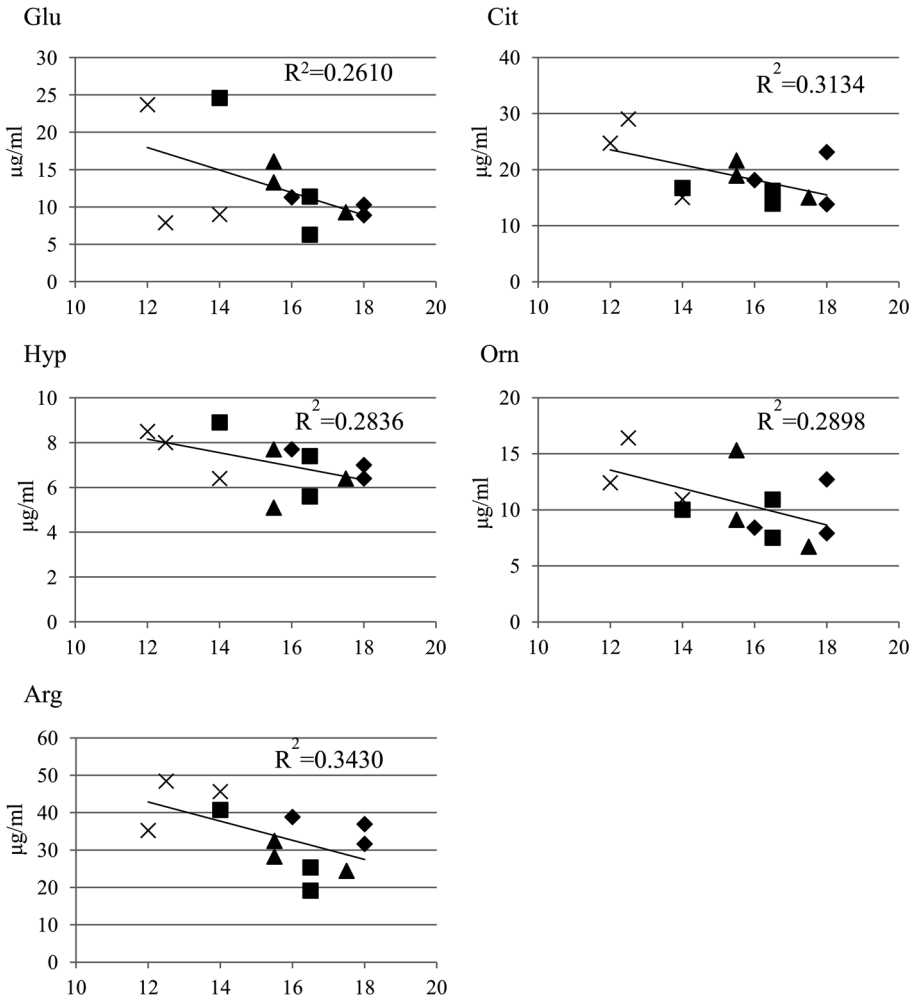

Fig. 3. Correlation between histological score and plasma amino acid concentrations post-ACLT. Scatter diagrams showing the correlation between histological score average and amino acid concentrations post-ACLT. The x-axis shows the histological score. The slope in the scatter plot represents the regression line. Arg was shown to have a significant correlation with the histological score ($P<0.05$), and Glu, Hyp, Cit and Orn showed a tendency to correlate with the histological score ($0.05<P<0.10$). ♦: C group, ■: F group, ▲: G group, ×: FG group.

blind studies in the early 1980s, investigators showed that oral glucosamine decreases pain and improves mobility in patients with OA and does not have any side effects [8, 15, 20, 23]. A dietary collagen supplement has also been suggested to improve joint pain and mobility [5, 6]. Warland *et al.* reported that the concentration of plasma amino acids varied after oral administration of fermented milk containing collagen hydrolysate [24]. Azuma *et al.* reported that oral administration of glucosamine changes the concentration of plasma amino acids in dogs [2]. We hypothesized that OA induces cartilage catabolism, and causes changes in plasma amino acid concentrations that correlate with the severity of OA. In fact, these amino acids have been reported to be involved in collagen synthesis and wound healing. Ala is one of the amino acids contained in almost all proteins. Alanine is degraded into glucose by the glucose-alanine cycle. The alanine decrease may suggest an increment in the glycogenesis of alanine induced by OA. Thr is one of essential amino acids, so it must be obtained by intake of foods. However, our results indicate that the C group also showed a tendency to increase. This might suggest the inhibition of degradation from Thr to succinyl-CoA, but the physiological role of this is not known. Met is one of the sulfur amino acids (SAAs). Organic sulfates are required for synthesis of GAGs, which area main components of the extracellular matrix. SAAs are the primary source of sulfur used in the synthesis of many key metabolic intermediates as well as GAGs [7]. Our results may suggest that the increase in plasma Met concentration is the compensatory change for cartilage degradation induced by OA. Arg is a basic amino acid that plays several pivotal roles in cellular physiology. Starting with the premise that arginine may become essential after surgery and wounding,

Seifer *et al.* showed that arginine is not essential for normal growth and development but becomes so in posttraumatic situations [1]. Arg enhances wound healing when given to arginine-depleted animals and when provided as supplementation to rats receiving recommended dietary arginine intake [25]. Oral arginine supplementation in human dermal wound healing enhances wound breaking strength [4]. Glu is one of the important stimulators of collagen synthesis [14]. OA severity or the correlation between the plasma Glu concentration post-ACLT and the histological assessment score might suggest consumption to compensate collagen synthesis from type II collagen degradation induced by OA. Hyp is abundant in collagen tissue. It also been reported that the Hyp concentration can be an index of collagen synthesis in plasma or wound fluid. There is a report that indicates a significant increase in the Hyp content of the granulation tissue indicating increased collagen turnover [3]. Our result might indicate the possibility that the plasma Hyp concentration suggests the collagen turnover induced by OA. Orn is the main metabolite of arginine in the urea cycle and shares many of the biopharmacologic effects of arginine. It has been shown that the effect of Orn was shown that the effect on wound healing is independent of the iNOS pathway [21]. Cit is also a precursor for arginine in the urea cycle. They may have a same tendency to correlate to progression of OA.

Some amino acids showed a significantly different plasma concentration between pre- and post-ACLT in all rabbits, regardless of the experimental group. Changes in the plasma concentrations of Ala, Thr and Met were significant. The increases and decreases in the plasma amino acid concentrations between pre- and post-ACLT regardless of administered materials might indicate that these changes were induced by the development of OA. As explained, progression of OA induced by the ACLT model after 4 weeks was slight and resulted in no obvious deformity in the joint surface macroscopically. Thus, if the increases and decreases in the concentrations of certain amino acids in plasma reflects the early stages of OA, the detection of these changes by routine blood examination should allow for the early diagnosis of OA prior to the development of clinical signs. This could make effective medical treatment aimed at delaying the progression of OA possible. The negative correlation between the histological assessment scores and the plasma concentrations of Arg post-ACLT was significant. This tendency was also observed for Glu, Hyp, Cit and Orn. Experimentally induced OA is usually evaluated on the basis of histological findings, whereas clinical OA is usually evaluated by symptoms such as pain. Clinical signs such as pain might be defined subjectively. For histological evaluation, we must collect materials, so it is impossible to perform continuous evaluations. For these perspectives, measurement of plasma amino acids, which correlate to both the condition of the articular cartilage and the effect of treatment, might enable continuous evaluation in an experimental group, and therefore allow for a more objective clinical evaluation.

Amino acids are components of collagen and are involved in the synthesis of collagen and wound healing [18]. There are no reports these amino acids were directly involved in cartilage chondroprotective effects or development of OA; however, our results indicate that these plasma amino acid concentrations are related to cartilage chondroprotective effects or the development of OA (Table 1), cartilage catabolism, or changes in metabolism. Two patterns of changes in the plasma amino acid concentrations were observed in our study. In one pattern, an increase or decrease in the concentration was observed between pre- and post-ACLT with the development of OA. In the second pattern, the plasma amino acid concentrations at post-ACLT were negatively correlated with the severity of OA Fig. 2. Measurement of the concentrations of the plasma amino acids might enable monitoring of the progression of OA. Therefore, it might also be useful for continuous evaluation of the efficacy of intervention or agents.

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