Evaluation of effects of oral administration of fish collagen peptide and glucosamine on experimentally cartilaginous damage model using serum biomarkers and plasma amino acid concentration

学位論文

大西　章弘

November 2012

CONTENTS

General introduction 1

Chapter 1 Abstract 7

Introduction 8

Materials and methods 9

Results 13

Discussion 15

Chapter 2 Abstract 21

Introduction 22

Materials and methods 23

Results 27

Discussion 29

Conclusions 35

Acknowledgement 36

References 37

General introduction

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 [14].

Cartilage is predominantly composed of collagen, with lesser amounts of proteoglycan (PG) and glycoprotein [12, 22]. 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 [14].

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 [17]. 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 [17]. 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 [9]. 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, a multifunctional ideal 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 [14], 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 [23]. 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 [8, 21, 31, 37].

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 [41]. 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 [5, 6].

Anterior cruciate ligament (ACL) injuries offer clinicians the opportunity to test pharmacological interventions aimed at slowing or stopping OA progression [20]. ACL transection (ACLT) has been widely used in different species as an experimental OA model. ACLT in rabbits leads to rapid development of OA [36, 44]. This animal model has been well characterized at both the macroscopic and microscopic levels, but data regarding its molecular characterization are lacking, as are biomarkers [10].

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. Similarly, we investigated whether plasma amino acids are correlated to the development and severity of OA by comparing plasma amino acid concentrations at pre- and post-ACLT.

Chapter 1

Evaluation of the chondroprotective effects of glucosamine and fish collagen peptide on a rabbit ACLT model using serum biomarkers

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 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- and post-ACLT were significantly correlated with OA severity. Administration of 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.

Introduction

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 [30]. 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.

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

1) 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, 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 3000. 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: Glucosamine was supplied by Yaizu Suisankagaku Industry Co., Ltd. The glucosamine we used was of >95% purity.

2) Methods

All experimental procedures were approved by the animal care and use committees of Tottori University and were 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. We 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 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. The limb was clipped and prepared for surgery

in a standard aseptic manner. A medial parapatellar incision was made through the skin and an arthrotomy performed. The patella was dislocated laterally and the knee 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, USA), 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 m*M* 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 anti-type II collagen mouse monoclonal antibody. The sections from each site and their averages were evaluated by criteria using a modified Mankin grading system as a reference (Table 1). Furthermore, we added a point to assess 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 kit (IBEX Technologies, Inc., Montreal, Quebec, Canada), and a QnE Hyaluronic Acid

(HA) ELISA kit (Biotech Trading Partners, Inc., Encinitas, Canada), 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 Kruskal-Wallis test. Post-ACLT associations between histological assessments and biomarker levels were made using Pearson's correlation coefficient test if normality of the groups were confirmed. Spearman rank-correlation coefficient was done 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 as same.

Results

1. 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. 1-A). 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 to the normal femoral condyle (Fig. 2-A, a, B, b). Decreasing in PG, type II collagen, and chondrocyte levels in the F, G, and FG groups (Fig. 2-C, c, D, d) 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 to 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 FG group and F, G group were also significant ($P < 0.05$, both) on the LFC compartment.

2. Serum biomarkers

The measurement values are shown in Fig. 3. There were no significant differences between pre-ACLT and post-ACLT values of serum KS, HA, or CS 846. In post-ACLT value, there were no significant differences between each group in serum KS, HA and CS 846.

3. Correlation between histological assessment and serum biomarkers

Correlations between histological assessment and serum biomarkers were evaluated in the post-ACLT concentration 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 value was significant for MFC and average scores and HA value ($P < 0.05$, both, Fig. 5-A).

Variations between pre- 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. 6-B).

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 chondroprotective effect, as evidenced by the amount of PG, type II collagen, and chondrocytes in the cartilaginous tissue compared to the C group. These statistical differences were also shown between FG and F, G group. This might suggest that administration both FCP and glucosamine show synergistic chondroprotective effect.

There were no significant differences between each group with post-ACLT in serum KS, HA and CS 846. This might suggest that it makes no significant difference between each group by administration of FCP, glucosamine or both.

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 serve a screening and monitoring test [40]. Our result didn't show that serum KS have significant difference between each group at post-ACLT or significant correlation with histological assessment score. It might our experimental period, 4 weeks is too short to show the serum KS as biomarker for early stage of OA. Therefore, to investigate the availability of KS needs more long experimental period.

HA is a constituent of 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 [11]. There is a report that showed the measurement of serum HA levels may be useful in assessing knee OA activity and determining predictive factors [34]. It was also reported that plasma HA levels in patients with OA directly correlated with the functional capacity and estimated burden of diseased cartilage [15]. 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 of OA, such as at 4 weeks. Administration of

both FCP and glucosamine might enhance the production of HA in synovial tissues [27, 37]. 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 [33]. 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 [32]. 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 up-regulated [19]. The correlation between 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 [27]. In the STR/ort mouse model of spontaneous OA, oral administration of collagen hydrolysate

could be potential interest as a disease-modifying agent for the prevention of degenerative joint disease [28]. Pro-Hyp has been identified in the blood after oral intake of collagen hydrolysate [29]. Nakatani et al. demonstrated the chondroprotective effect of Pro-Hyp under pathological conditions in mouse in vivo and in vitro models [24]. Moreover, it has been suggested that food-derived Hyp-containg peptides can affect the PGs and the morphological changes associated with OA cartilage, which might be mediated by stimulation of HA production in the synovium [28].

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 [39]. Oral glucosamine has the effect of restoring GAG in damaged cartilage, but its mechanisms of action are unknown [26]. On the other hand, glucosamine was reported to have an effect on type II collagen synthesis [23]. 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 [23]. In addition, glucosamine has a positive effect on subchondral bone turnover, structure, and mineralization in the early stages of experimental OA [42]. It was reported that exogenous glucosamine can increase HA production of the synovial

tissue [37]. This might also be useful for preventing OA development or progression OA.

From our present results, oral administration of FCP and/or glucosamine effectively controlled cartilage degradation in an ACLT model. The estimation of various biomarkers for arthritis will be useful for estimating the progression of cartilaginous degradation; in this study, the estimation of HA and CS 846 might be useful for monitoring OA progression because the HA and CS 846 levels detected correlated with the histological findings in our study. 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 concentrations of biomarkers measured can be used in addition to histological findings to evaluate cartilage injury.

Chapter 2

Correlation of plasma amino acid concentrations and chondroprotective effects of glucosamine and fish collagen peptide on the development of osteoarthritis

Abstract

The aim of this study was to investigate the correlation of plasma amino acids concentration and development or severity of osteoarthritis (OA). 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. Blood was collected before surgery (pre-ACLT) and before euthanasia (post-ACLT) for plasma amino acids measurement. Plasma amino acids concentrations were measured by HPLC. We evaluated OA severity both macroscopically and histologically. Macroscopic evaluation showed mildly eroded condylar surfaces in the C group. Histologic findings were significantly different from the FG and other groups. Changes of the amino acid concentrations between pre- and post-ACLT in all rabbits were significant difference in alanine, threonine, methionine. Correlation between histological assessment and each plasma amino acids at post-ACLT was significant in

arginine, and showed the tendency in glutamate, hydroxyproline, citrulline, ornithine. Our results suggest that measurement of plasma amino acids concentrations might take it possible to monitoring progress of OA and contiunuous evaluation of efficacy of intervention or agents.

Introduction

Nutraceuticals are candidates for long-term prevention of chronic disease, such as OA [17]. 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. There are reported that oral administration of glucosamine and collagen hydrolysate varied the concentration of plasma amino acids [2, 41]. Therefore, we hypothesized that these supplemental chondroprotective mechanisms are related to plasma amino acid. We also investigated whether plasma amino acids are correlated to the development and severity of OA by comparing plasma amino acid concentrations at pre- and post-ACLT.

Materials and methods

1) Materials

Animals: Twelve clinically healthy rabbits (Japanese albino, females; 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 of acclimatization to the laboratory environment.

FCP: Collagen is extracted from skins of 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 is 3,000. The major amino acids constituting FCP are glycine (24.6% of dry matter), glutamic acid (10.8%), proline (10.6%), and alanine (9.5%).

Glucosamine: Glucosamine was supplied by Yaizu Suisankagaku Industry Co., Ltd. The glucosamine we used was >95% purity.

2) Methods

All experimental procedures were approved by the animal care and use committes of Tottori University, and were 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 four group, namely the control (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. We administrated FCP and glucosamine after surgery and continued for 4

weeks.

Oral administration of Glucosamine and FCP

The control group had tap water to drink freely. 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 the F group, 1.0 g of powdered FCP/day was dissolved into half average daily quantity of drinking water for each rabbit, and this water was orally administered. The G group received 1.0 g of glucosamine and FG group received 1.0 g of FCP and 1.0 g of glucosamine daily as same as F group.

ACLT surgery

An analgesic (xylazine hydrochloride, 10 mg/kg) was administrated as premedication. After sedation, induction anesthesia was performed in a box by inhalation of mixture of 5 % isoflurane in oxygen. Anesthesia was maintained by inhalation of mixture of 3 % isoflurane in oxygen using a mask. The limb was clipped and prepared for surgery in a standard aseptic manner. A medial arthrotomy was performed on the femopatellar joint to permit transection of the ACL. The joint capsule with subcutaneous tissue was closed by interrupted sutures with absorbable suture (3-0 Maxon, Johnson & Johnson, New

Jersey) and skin incision was closed interruptedly with stainless suture (Wire Spool, Kirikan Youkou, Tokyo). During the week following the operation, 10 mg/kg of oxytetracycline (terramycin, Pfizer, Tokyo) was subcutaneously administered twice a day to prevent infection.

Post-mortem examination

At 4 weeks after surgery, the rabbits were euthanized by an intravenously administered overdose (80 mg/kg) of pentobarbital (Nembutal; Dainippon Pharmaceutical Co., Osaka). The knee joints were opened and were macroscopically observed at the operated site for the injured cartilage.

Evaluation of injured sites by histological findings

Histological assessment of cartilage: Histologic assessment was performed on the femur of rabbit in the each group. The femoral articular surfaces were fixed in 10 % neutral buffered formalin. Tissue blocks were decalcified with 14 % EDTA in 10 m*M* phosphate buffer (pH 7.4), dehydrated through graded alcohols, rinsed in toluol, embedded in paraffin. Six-micrometer sections were cut at a standard site centrally in the medial femoral condyle (MFC) and lateral femoral condyle (LFC). This sections

were stained with Safranin O-fast green, hematoxylin-eosin-saffron. And immunohistochemical staining was performed with anti-type II collagen mouse monoclonal antibody. These sections from each site and their average were evaluated by criteria using modified Mankin grading system as reference (Table 1). Furthermore, we added a point to assess the loss of type II collagen. The sites of histological assessment were divided into two parts, MFC, LFC and we evaluated each sites and their average.

Measurement of plasma amino acid concentrations

Blood was collected at before surgery (pre-ACLT) and before euthanasia (post-ACLT) using heparin as an anti-coagulant. The blood was centrifuged at 3,000 rpm for 10 min, and the plasma was then separated promptly and frozen at -80 ℃ until measurement of plasma amino acid concentrations. Plasma were mixed with equal volumes of 3% (w/w) sulfosaliclic acid, and left to stand at 4 ℃ for 1 h. Samples were then centrifuged (4 ℃, 15 min, 1,500 rpm), and precipitated protein was removed. The amino acid concentrations were measured by an automatic amino acid analyzer (JLC-500/V2, Amino Tac; JEOL, Tokyo, Japan). The amino acids measured are listed in Table 3.

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 amino acids' concentrations between animal groups were analyzed with one-way ANOVA and multiple comparisons (Scheffe's F test). Post-ACLT associations between histological assessments on average and biomarker levels were made using Pearson's correlation coefficient test if normality of the groups were confirmed. Spearman rank-correlation coefficient was done if normality of the groups was not identified. Associations between histological assessments on average and changes in biomarker levels (post-ACLT value – pre-ACLT value) were also identified as same.

Results

1. Macroscopic and histological findings and assessment scores

At the 4 week post-surgery, there is some mild erosion on the condyle surfaces in the C group, however the difference was not recognized macroscopically between each group. In histological findings in C group at the 4 week post-surgery, the surface of femoral condyle was covered by fibrous tissue instead of cartilaginous tissue. Decreasing of chondrocytes, proteoglycan and type II collagen were clear compare to

the normal femoral condyle in the Fig 7. In the F, the G, and the FG group (Fig 8) decreasing of proteoglycan, type II collagen, and chondrocytes were mild. These histological findings were almost similar in 3 zones.

The total histological assessment scores of all sites (MFC, LFC and average) were shown in Fig 9. The average score was estimated as follows: (MFC score + LFC score)/2. There are trend to decrease the loss of Safranin O staining and type II collagen immunostaining by administration FCP and glucosamine compare to the C group. The results indicated administration FCP and glucosamine prevented articular cartilage degradation by ACLT.

There were significant lower mean score for total of global score in the LFC and average compartment in the FG group compared with the C group ($P = 0.047, 0.049$ respectively).

2. Amino acid concentrations

Changes of the amino acid level between pre-ACLT and post-ACLT, or change of plasma amino acid concentration by osteoarthritis induced by ACLT in all rabbits were significant difference in Ala, Thr, Met (Fig 10; ◆, C group; ■, F group; ▲, G group; ×, FG group).

3. Correlation between histological assessment and amino acid concentrations

Correlation between histological assessment and each plasma amino acids at post-ACLT was evaluated. The each average score was used as histological assessment score.

The significant correlation was shown in Arg, and this tendency was also shown in Glu, Hyp, Cit and Orn (Fig 11; ◆, C group; ■, F group; ▲, G group; ×, FG group). These amino acids found significant negative correlation between histological assessment scores and values of the concentrations at post ACLT.

Discussion

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 concentration of certain amino acids changed during the development of OA, or the concentration of these amino acids was negatively correlated to the severity of OA. From the results of the histological assessment score, the FG group, compared to 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 ulceration was noted after 4

weeks [44], and therefore, our experimental OA represented the early stages of disease. The effects of glucosamine for OA are shown by inhibiting the degradation and stimulating the synthesis of proteoglycan [13, 16]. In addition, glucosamine is excepted an anti-inflammatory action [18]. Collagens are reported the effects stimulating type II collagen and proteoglycan synthesis, as well as aggrecan expression by chondrocytes [27]. Collagens are also shown the effects of preventing chondrocyte differentiation into mineralized chondrocytes [24]. Both glucosamine and collagen are not reported the correct mechanism indicating the effects. But the effects of both have some similarity. In addition, only FG group has significant difference against C group in histological findings. Therefore, we evaluated both administration both collagen and glucosamine has the synergetic effect. There are some reports indicating that collagen and glucosamine are useful for OA patients. In several European double-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, 13, 21, 38]. 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 [41]. 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 correlate to 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 amino acid contained in almost all proteins. Alanine is degraded into glucose by glucose-alanine cycle. The alanine decrease may suggest increment the glycogenesis of alanine induced by OA. Thr is one of essential amino acids, so they had to be taken by foods. But our results indicate the C group also showed the increase tendency. This might suggest that the inhibition of degradation from Thr to succinyl-CoA but physiological role is not known. Met is one of sulfur amino acids (SAA). Organic sulfate are required for the GAG synthesis, main components of the extracellular matrix. SAA are primary source of sulfur used in the synthesis of many key metabolic intermediates as well as GAG [7]. Our results may suggest that the increase of 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 post-traumatic situations [1]. Arg enhances wound healing when given to arginine-depleted animals and when provided as

supplementation to rats receiving recommended dietary arginine intake [43]. Oral arginine supplementation in human dermal wound healing and enhances wound breaking strength [4]. Glu is one of important stimulator of collagen synthesis [18]. The correlation between plasma Glu concentration at post-ACLT and histological assessment score, or OA severity might suggest consumption by compensate collagen synthesis from type II collagen degradation induced by OA. Hyp is abundant in collagen tissue. Hyp is also reported that Hyp concentration can be 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 plasma Hyp concentration suggest 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. Orn was shown that the effect on wound healing is independent of iNOS pathway [35]. 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-ACLT and post-ACLT in all rabbits, regardless of the experimental group. Changes in plasma concentration of Ala, Thr, and Met were significant. The increase and

decrease of plasma amino acid concentrations between pre-ACLT 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 increase and decrease in the concentration 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 at 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 makes impossible to continuous evaluation. For these points, the measurement of plasma amino acids, which correlates to both the condition of the articular cartilage and the effect of treatment, might enable a 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 [25]. There are no reports that 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, cartilage catabolism, or changes in metabolism. Two patterns of changes in the plasma amino acid concentrations were observed in our study. One pattern was where an increase or decrease in the concentration was observed between pre- and post-ACLT by the development of OA. The second pattern was where the plasma amino acid concentrations at post-ACLT were negatively correlated to the severity of OA. 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.

Conclusions

From our results, oral administration of FCP and/or glucosamine has chondroprotective effects in an ACLT model. The measurement of serum biomarkers, especially HA and CS 846 in our experiment might be useful for monitoring progression of OA. In plasma amino acids, our results indicate that some amino acid concentrations are related to development of OA and correlated to severity of OA. The measurement of serum biomarkers or plasma amino acids might enable monitoring of the progression of OA. Moreover, it might be useful for continuous evaluation of the efficacy of intervention or agents.

Acknowledgement

The author thank Prof. Saburo Minami (Department of veterinary, Tottori University) for suggesting that conception and design for these experiments; Prof. Yoshiharu Okamoto, Prof. Tomohiro Imagawa, Dr. Takeshi Tsuka and Dr. Yoshiharu Matahira (Yaizu Suisankagaku Industry, Ltd.) encouragement during the early days of the study; Dr. Tomohiro Osaki for suggesting that conception and design for these experiments and preparing the original manuscript. The author wish to thank the Yaizu Suisankagaku Co. Ltd. For kindly supply of fish collagen peptide and glucosamine.

Reference

1. Albina, J. E., Millis, C. D., Barbul, A., Thirkill, C. E., Henry, W. L. Jr., Mastrofrancesco, B. and Caldwell, M. D. 1988. Arginine metabolism in wounds. *Am. J. Physiol.* **254**: 459-467.

2. Azuma, K., Osaki, T., Tsuka, T., Imagawa, T., Okamoto, Y., Takamori, Y. and Minami, S. 2011. Effects of oral glucosamine hydrochloride administration on plasma free amino acid concentrations in dogs. *Mar. Drugs* **9**: 712-718.

3. Badiu, D. L., Luque, R., Dumitrescu, E., Craciun, A. and Dinca, D. 2010. Amino acids from Mytilus galloprovincialis (L.) and Rapana venosa molluscs accelerate skin wounds healing via enhancement of dermal and epidermal neoformation. *Protein J.* **29**: 81-92.

4. Barbul, A., Lazarou, S. A., Efron, D. T., Wasserkrug, H. L. and Efron, G. 1990. Arginine enhances wound healing and lymphocyte immune responses in humans. *Surgery* **108**: 331-337.

5. Benito-Ruiz, P., Comacho-Zambrano, M. M., Carrilo-Arcentales, J. N., Mestanza-Peralta, M. A., Vallejo-Flores, C. A., Vargas-Lopez, S. V., Villacis-Tamayo, R. A. and Zurita-Gavilanes, L. A. 2009. A randomized controlled

trial on the efficacy and safety of a food ingredient, collagen hydrolysate, for improving joint comfort. *Int. J. Food. Sci. Nutr. (Suppl.)* **60**: 99-113.

6. Clark, K. L., Sebastianelli, W., Flechsenhar, K. R., Aukermann, D. F., Meza, F., Millard, R.L., Deitch, J.R., Sherbondy, P. S. and Albert, A. 2008. 24-Week study on the use of collagen hydrolysate as a dietary supplement in athletes with activity-related joint pain. *Curr. Med. Res. Opin.* **24**: 1485-1496.
7. Cordoba F, Nimni ME. Chondroitin sulfate and other sulfate containing chondroprotective agents may exhibit their effects by overcoming a deficiency of sulfur amino acids. Osteoarthritis Cartilage 2003; 11: 228-30.
8. Crolle, G. and D'Este, E. 1980. Glucosamine sulphate for the management of arthrosis: a controlled clinical investigation. *Curr. Med. Res. Opin.* **7**: 104-109.
9. De Ceuninck, F. Sabatini, M. and Pastoureau, P. 2011. Recent progress toward biomarker identification in osteoarthritis. *Drug Discov. Today* [Equp ahead of print]
10. Duclos, M. E., Roualdes, O., Cararo, R., Rousseau, J. C., Roger, T. and Hartmann, D. J. 2010. Significance of the serum CTX-II level in an osteoarthritis animal model: a 5-month longitudinal study. *Osteoarthritis Cartilage* **18**: 1467-1476.
11. Elliott, A. L., Kraus, V. B., Luta, G., Stabler, T., Renner, J. B., Woodard, J., Dragomir, A. D., Helmick, C. G., Hochberg, M. C. and Jordan, J. M. 2005. Serum

hyaluronan levels and radiographic knee and hip osteoarthritis in African Americans and Caucasians in the Johnston Country Osteoarthritis Project. *Arthritis Rheum.* **52**: 105-111.

12. Eyre, D.R. 2004. Collagenous and cartilage matrix homeostasis. *Clin. Orthop. Relat. Res.* **427**: 118-122.
13. Fenton, J. I., Chlebek-Brown, K. A., Peters, T. L., Caron, J. P. and Orth, M. W. 2000. Glucosamine HCl reduces equine articular cartilage degradation in explant culture. *Osteoarthritis Cartilage* **8**: 258-265.
14. Garvican, E. R., Vaughan-Thomas, A., Innes, J. F. and Clegg, P. D. 2010. Biomarkers of cartilage turnover. Part 1: Markers of collagen degradation and synthesis. *Vet. J.* **185**: 36-42.
15. Goldberg, R. L., Huff, J. P., Lenz, M. E., Glickman, P., Katz, R. and Thonar, E. J.1991. Elevated plasma levels in patients with osteoarthritis and rheumatoid arthritis. *Arthritis Rheum.* **34**: 799-807.
16. Gouze, J. N., Bordji, K., Gulberti, S., Terlain, B., Netter, P., Magdalou, J., Fournel-Giigleux, S. and Ouzzine, M. 2001. Interleukin-1β down-regulates the expression of glucronosyltransferase I, a key enzyme priming glycosaminoglycan

biosynthesis: influence of glucosamine on interleukin-1β –mediated effects in rat chondrocytes. *Arthritis Rheum.* **44**. 351-360.

17. Henrotin, Y., Lambert, C., Couchourel, D., Ripoll, C. and Chiotelli, E. 2008. Nutraceuticals: do they represent a new era in the management of osteoarthritis? – a narrative review from the lessons taken with five products. *Osteoarthritis Cartilage* **19**: 1-21.
18. Karna, E., Miltyk, W., Wolczynski, S. and Palka, J. A. 2001. The potential mechanism for glutamine-induced collagen biosynthesis in cultured human skin fibroblasts. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **130**: 23-32.
19. Lohmander, L. S., Ionescu, M., Jugessur, H. and Poole, A. R. 1999. Changes in joint cartilage aggrecan after knee injury and in osteoarthritis. *Arthritis Rheum.* **42**: 534-44.
20. Lohmander, L. S., Ostenberg, A., Englund, M. and Roos, H. 2004. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. *Arthritis Rheum.* **50**: 3145-3152.

21. Lopes, Vaz, A. 1982. Double-blind clinical evaluation of the relative efficacy of ibuprofen and glucosamine sulphate in the management of osteoarthrosis of the knee in out-patients. *Curr. Med. Res. Opin.* **8**: 145-149.

22. Maroudas, A., Bayliiss, M. T. and Venn, M. F. 1980. Further studies on the composition of human femoral head cartilage. *Ann. Reum. Dis.* **39**: 514-523.

23. Naito, K., Watari, T., Furuhata, A., Yomogida, S., Sakamoto, K., Kurosawa, H., Kaneko, K. and Nagaoka, I. 2010. Evaluation of the effect of glucosamine on an experimental rat osteoarthritis model. *Life Sci.* **86**: 538-543.

24. Nakatani, S., Mano, H., Sampei, C., Shimizu, J. amd Wada, M. 2009. Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse articular cartilage in vitro and in vivo. *Osteoarthritis Cartilage* **17**: 1620-1627.

25. Ng, K. W., DeFrancis, J. G., Kugler, L. E., Kelly, T. A., Ho, M. M., O'Conor, C. J., Ateshian, G. A. and Hung, C. T. 2008. Amino acids supply in culture media is not a limiting factor in the matrix synthesis of engineered cartilage tissue. *Amino Acids* **35**: 433-438.

26. Oegema, T. R. Jr, Deloria, L. B., Sandy, J. D. and Hart, D. A. 2002. Effect of oral glucosamine on cartilage and meniscus in normal and chymopapain-injected knees of young rabbits. *Arthritis Rheum.* **46**: 2495-2503.

27. Oesser, S. and Seifert, J. 2003. Stimulation of type II collagen biosynthesis and secretion in bovine chondrocytes cultured with degraded collagen. *Cell Tissue Res.* **311**: 393-399.

28. Ohara, H., Iida, H., Ito, K., Takeuchi, Y. and Nomura, Y. 2010. Effects of Pro-Hyp, a collagen hydrolysate-derived peptide, on hyaluronic acid synthesis using in vito cultured synovium cells and oral ingestion of collagen hydrolysates in a guinea pig model of osteoarthritis. *Biosci. Biotechnol. Biochem.* **74**: 2096-2099.

29. Ohara, H., Matsumoto, H., Ito, K., Iwai, K. and Sato, K. 2007. Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. *J. Agric. Food Chem.* **55**: 1532-1535.

30. Okumura, M., Tagami, M. and Fujinaga, H. 1998. Measurement of serum and synovial fluid keratan sulphate and antibody to collagen type II in equine osteoarthritis. *Zentralbl. Veterinarmed. A.* **45**: 513-516.

31. Pujalte, J. M., Llavore, E. P. and Ylescupidez, F. R. 1980. Double-blind clinical evaluation of oral glucosamine sulphate in the basic treatment of osteoarthrosis. *Curr. Med. Res. Opin.* **7**: 110-114.

32. Rizkalla, G., Reiner, A., Roqoch, E. and Poole, A. R. 1992. Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. *J. Clin. Invest.* **90**: 2268-2277.

33. Rousseau, J. C. and Delmas, P. D. 2007. Biological markers in osteoarthritis. *Nat. Clin. Pract. Rheumatol.* **3**: 346-356.

34. Sharif, M., George, E., Shepstone, L., Knudson, W., Thonar, E. J., Crushnaghan, J. and Dieppe, P. 1995. Serum hyaluronic acid level as a predictor of disease progression in osteoarthritis of the knee. *Arthritis Rheum.* **38**: 760-767.

35. Shi, H. P., Fishel, R. S., Efron, D. T., Williams, J. Z., Fishel, M. H. amd Barbul, A. 2002. Effect of supplemental on wound healing. J. Surg. Res. 106: 299-302.

36. Takahashi, K., Hashimoto, S., Kudo, T., Hirasawa, Y., Lotz, M. and Amiel, D. 2000. Effect of hyaluronan on chondrocyte apoptosis and nitric oxide production in experimentally induced osteoarthritis. *J. Rheumatol.* **27**: 1713-1720.

37. Uitterlinden, E. J., Koevoet, J. L., Verkoelen, C. F., Bierma-Zeinstra, S. M., Jahr, H., Weinans, H., Verhaar, J. A. and van Osch G. J. 2008. Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants. *BMC Muscockelet. Disord.* **9**: 120.

38. Vajaradul, Y. 1981. Double-blind clinical evaluation of intra-articular glucosamine in outpatients with gonarthrosis. *Clin. Ther.* **3**: 336-343.

39. Vidal, Y., Plana, R. R., Bizzarri, D. and Rovati, A. L. 1978. Articular cartilage pharmacology. I. In vitro studies on glucosamine and nonsteroidal anti-inflammatory drugs. *Pharmacol. Res. Commun.* **10**: 557-569.

40. Wakitani, S., Nawata, M., Kawaguchi, A., Okabe, T., Takaoka, K., Tsuchiya, T., Nakaoka, R., Masuda, H. and Miyawaki, K. 2007. Serum keratin sulfate is a promising marker of early articular cartilage breakdown. *Rheumatology (Oxford)* **46**: 1652-1656.

41. Walrand, S., Chiotelli, E., Noirt, F., Mwewa, S. and Lassel, T. 2008. Consumption of a functional fermented milk containing collagen hydrolysate improves the concentration of collagen-specific amino acids in plasma. *J. Agric. Food Chem.* **56**: 7790-7795.

42. Wang, A. X., Laverty, S., Dumitriu, M., Plaas, A. and Grynpas, M. D. 2007. The effects of glucosamine hydrochloride on subchondral bone changes in an animal model of osteoarthritis. *Arthritis Rheum.* **56**: 1537-1548.

43. Witte, M. B. and Barbul, A. 2003. Arginine physiology and its implication for wound healing. *Wound Repair Regen.* **11**: 419-23.

44. Yoshioka, M., Coutts, R. D., Amiel, D. and Hacker, S. A. 1996. Characterization of a model of osteoarthritis in the rabbit knee. *Osteoarthritis Cartilage* **4**: 87-98.

Table 1. Criteria (grading) for histological evaluation

Safranin O-Fast green staining

0 = Uniform staining throughout articular cartilage

1 = Loss of staining in the superficial zone for less than half the length of the condyle

2 = Loss pf staining in the superficial zone for half or more the length of the condyle

3 = Loss of staining in the superficial and middle zones for less than half the length of the condyle

4 = Loss of staining in the superficial and middle zones for half or more the length of the condyle

5 = Loss of staining in all 3 zones for less than half the length of the condyle

6 = Loss of staining in all 3 zones for half or more the length of the condyle

Type II collagen immunostaining

0 = Uniform staining throughout articular cartilage

1 = Loss of staining in the superficial zone for less than half the length of the condyle

2 = Loss pf staining in the superficial zone for half or more the length of the condyle

3 = Loss of staining in the superficial and middle zones for less than half the length of the condyle

4 = Loss of staining in the superficial and middle zones for half or more the length of the condyle

5 = Loss of staining in all 3 zones for less than half the length of the condyle

6 = Loss of staining in all 3 zones for half or more the length of the condyle

Chondrocyte loss

0 = No decrease in cells

1 = Minimal decrease in cells

2 = Moderate decrease in cells

3 = Marked decrease in cells

4 = Very extensive decrease in cells

Structure

0 = Normal

1 = Surface irregularities

2 = 1-3 Superficial clefts

3 = >3 Superficial clefrs

4 = 1-3 Clefts extending into the middle zone

5 = >3 Clefts extending into the middle zone

6 = 1-3 Clefts extending into the deep zone

7 = >3 Clefts extending into the deep zone

8 = Clefts extending into calcified cartilage

Table 2. Histological assessment scores of each site (medial femoral condyle [MFC, lateral femoral condyle [LFC] and average).

		Safranin O-Fast green staining	Type II collagen immunostaining	Chondrocyte loss	Structure	Global score
C group	MFC	6.00 ± 0.00	5.67 ± 0.58	3.67 ± 0.58	2.00 ± 0.00	17.33 ± 1.15
	LFC	6.00 ± 0.00	5.67 ± 0.58	3.67 ± 0.58	2.00 ± 0.00	17.33 ± 1.15 *
	Average	6.00 ± 0.00	5.67 ± 0.58	3.67 ± 0.58	2.00 ± 0.00	17.33 ± 1.15 **
F group	MFC	5.33 ± 1.15	5.00 ± 0.00	3.67 ± 0.58	2.00 ± 0.00	16.00 ± 1.73
	LFC	5.33 ± 0.58	5.00 ± 0.00	3.67 ± 0.58	1.33 ± 0.58	15.33 ± 1.15 *
	Average	5.33 ± 0.76	5.00 ± 0.00	3.67 ± 0.58	1.67 ± 0.29	15.67 ± 1.44
G group	MFC	6.00 ± 0.00	5.33 ± 0.58	3.67 ± 0.58	2.00 ± 0.00	16.67 ± 1.15
	LFC	6.00 ± 0.00	5.33 ± 0.58	3.33 ± 0.58	1.33 ± 0.58	15.67 ± 1.15 *
	Average	6.00 ± 0.00	5.33 ± 0.58	3.50 ± 0.50	1.67 ± 0.29	16.17 ± 1.15
FG group	MFC	4.67 ± 1.15	4.33 ± 0.58	2.68 ± 0.58	1.67 ± 0.58	13.33 ± 1.53
	LFC	4.67 ± 0.58	4.00 ± 0.00	2.33 ± 0.58	1.33 ± 0.58	12.33 ± 0.58 *
	Average	4.67 ± 0.76	4.17 ± 0.29	2.50 ± 0.50	1.50 ± 0.50	12.83 ± 1.04 **

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 aand 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

($P < 0.05$).

Table 3. Amino acids measured in this study

Essential amino acids (EAA)	**Nonessential amino acids (NEAA)**
Threonine (Thr)	Asparate (Asp)
Valine (Val)	Serine (Ser)
Methionine (Met)	Arginine (Arg)
Isoleucine (Ile)	Glutamate (Glu)
Leucine (Leu)	Glutamine (Gln)
Phenylalanine (Phe)	Glycine (Gly)
Lysine (Lys)	Alanine (Ala)
Histidine (His)	Tyrosine (Tyr)
Tryptophan (Trp)	Proline (Pro)
	Citrulline (Cit)
	Ornithine (Orn)
	Hydroxyproline (Hyp)

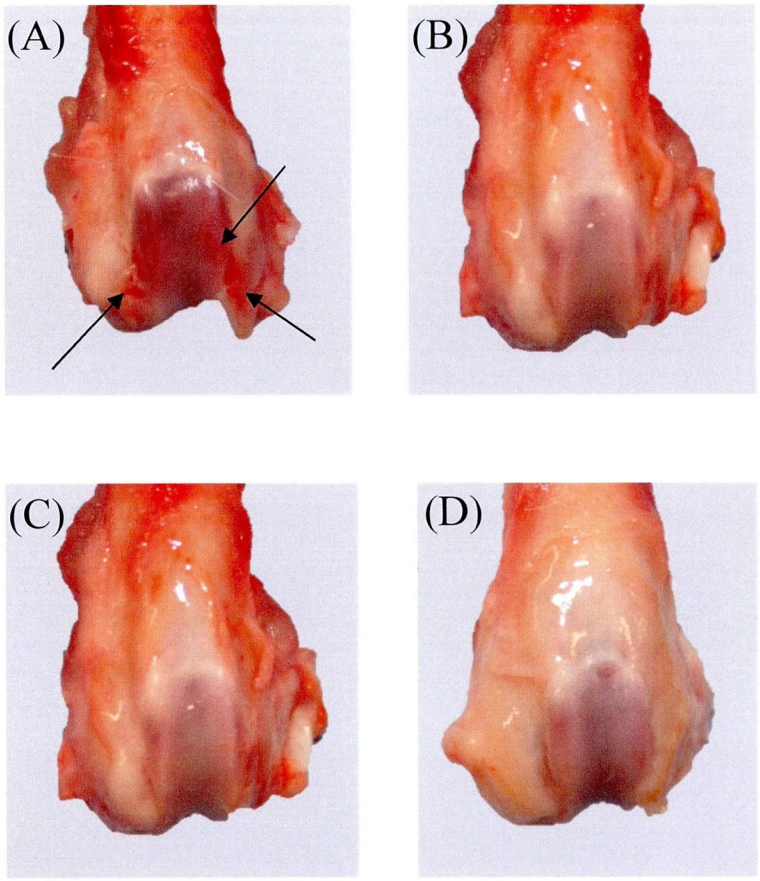

Fig. 1. Macroscopic findings at 4 weeks post-surgery

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).

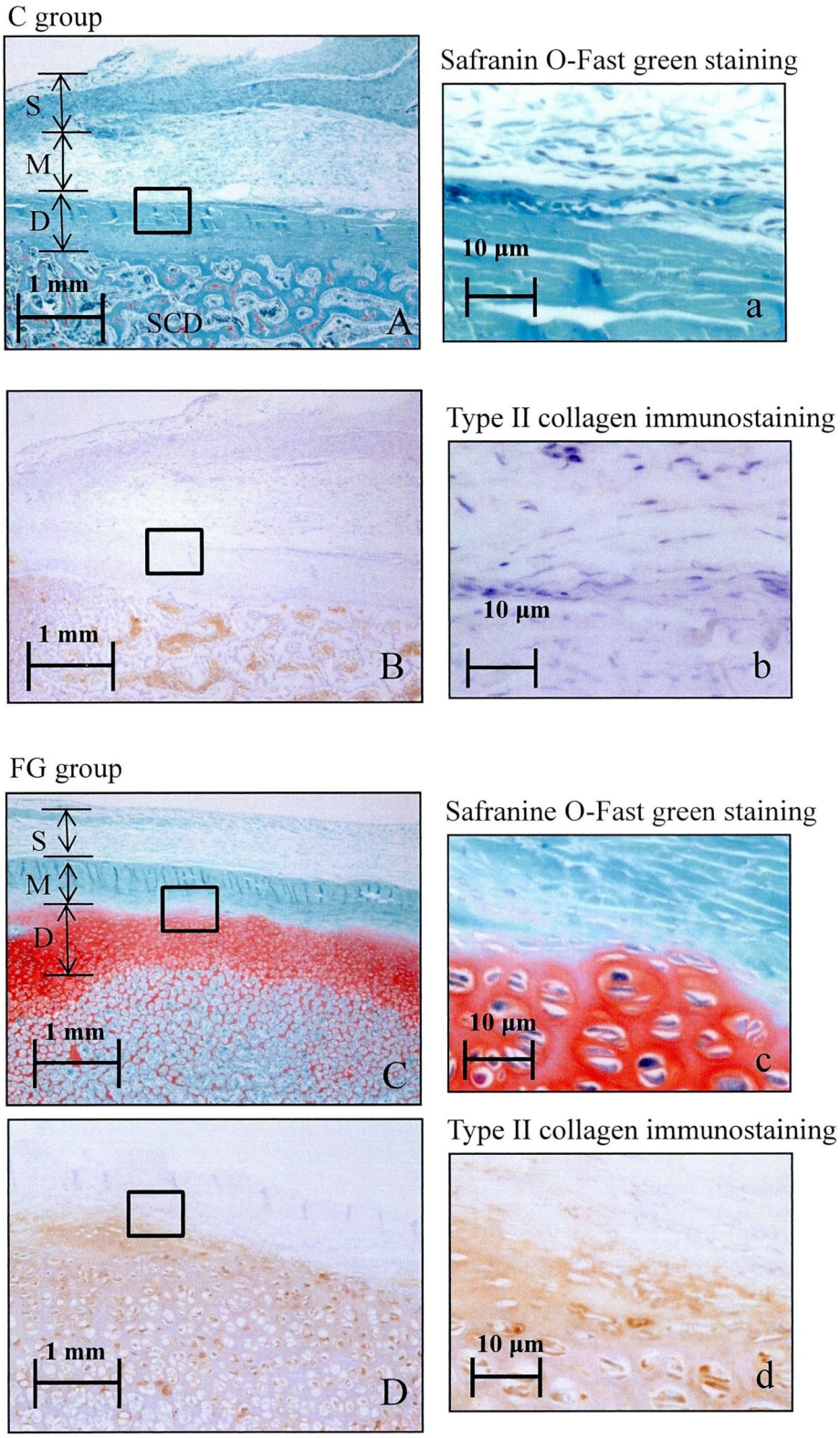

Fig. 2. Histological findings of the lateral femoral condyle in control and FG group 4 weeks at post-surgery.

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 Fig. 2A, 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 Fig. 2B. 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 Fig. 2C. 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 Fig. 2D showing staining of type II collagen around the cells in the D zone.

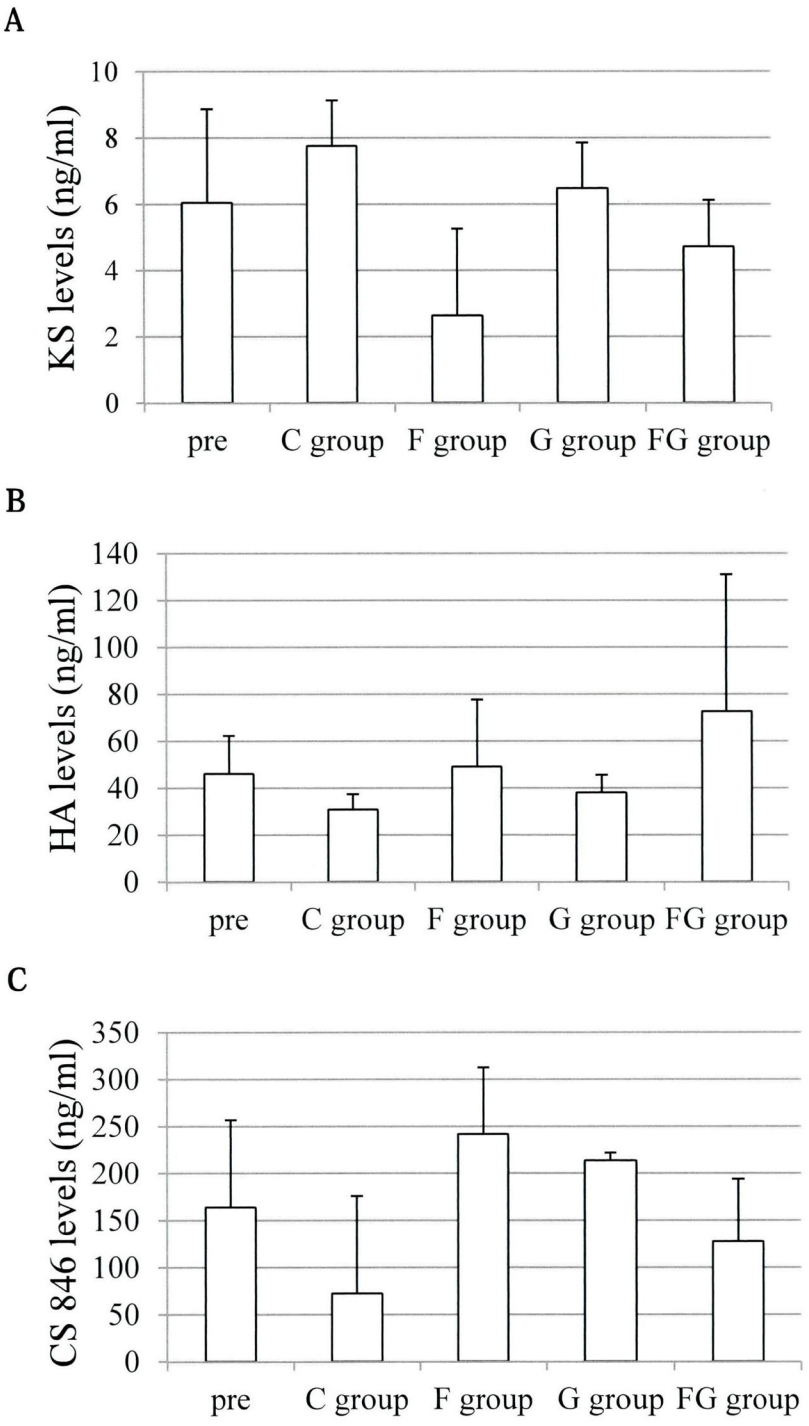

Fig. 3. Pre- and post-anterior cruciate ligament transection (ACLT) serum biomarker concentrations (A, keratan sulfate [KS]; B, hyaluronic acid [HA]; C, chondroitin sulfate 846 epitope [CS 846]).

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.

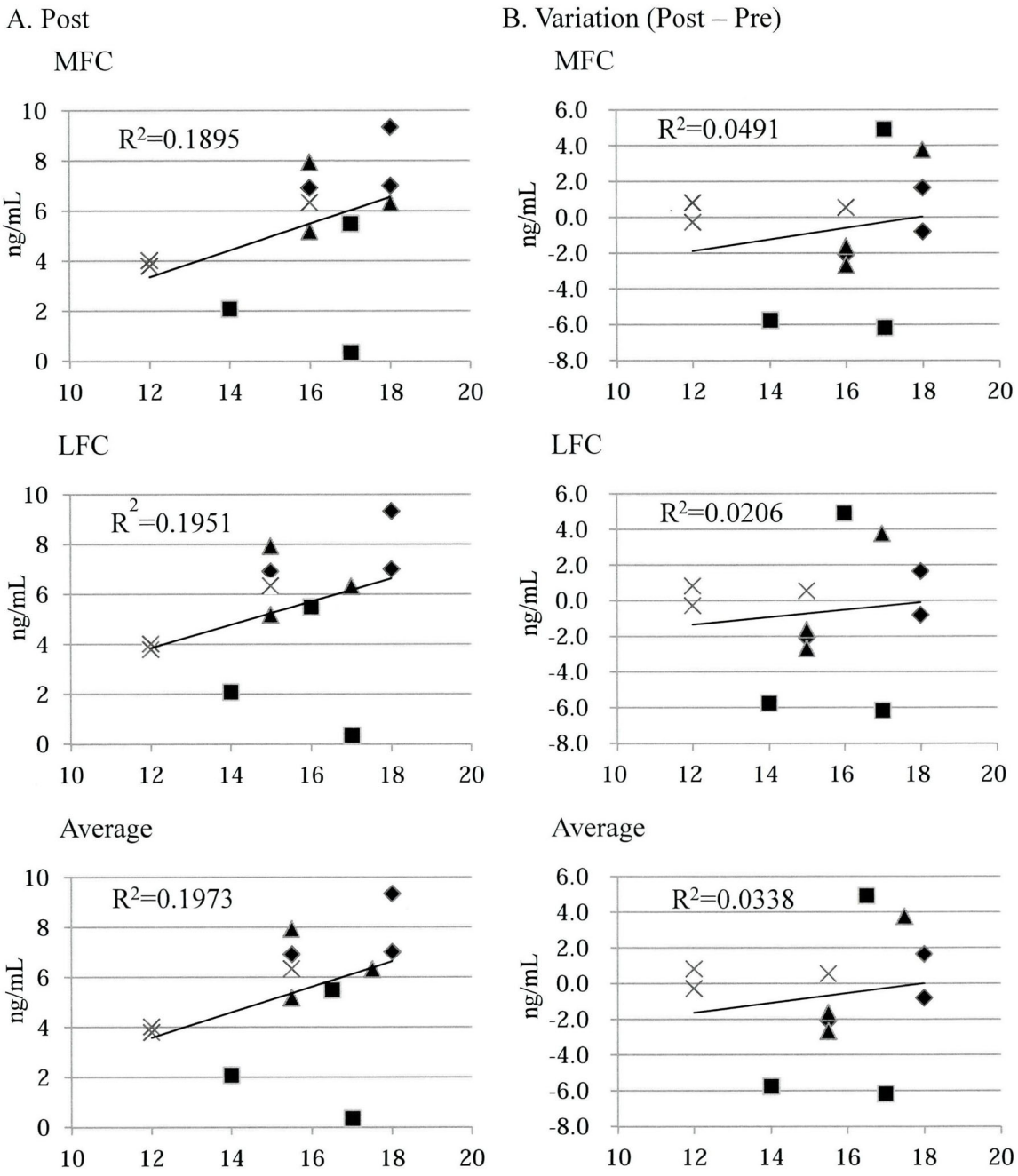

Fig. 4. Correlation between histological score and serum KS concentrations

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.

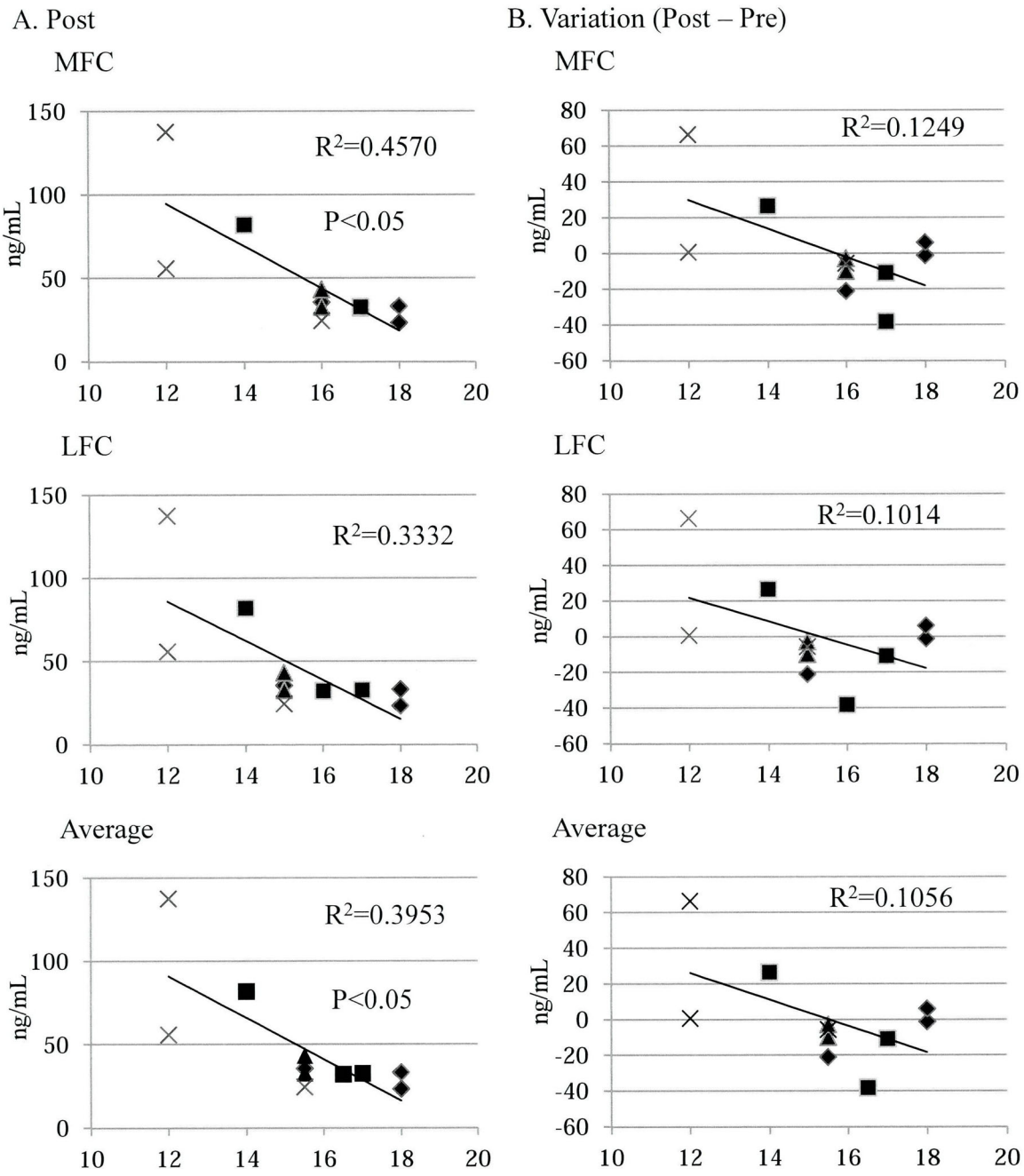

Fig. 5. Correlation between histological score and serum HA concentrations

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.

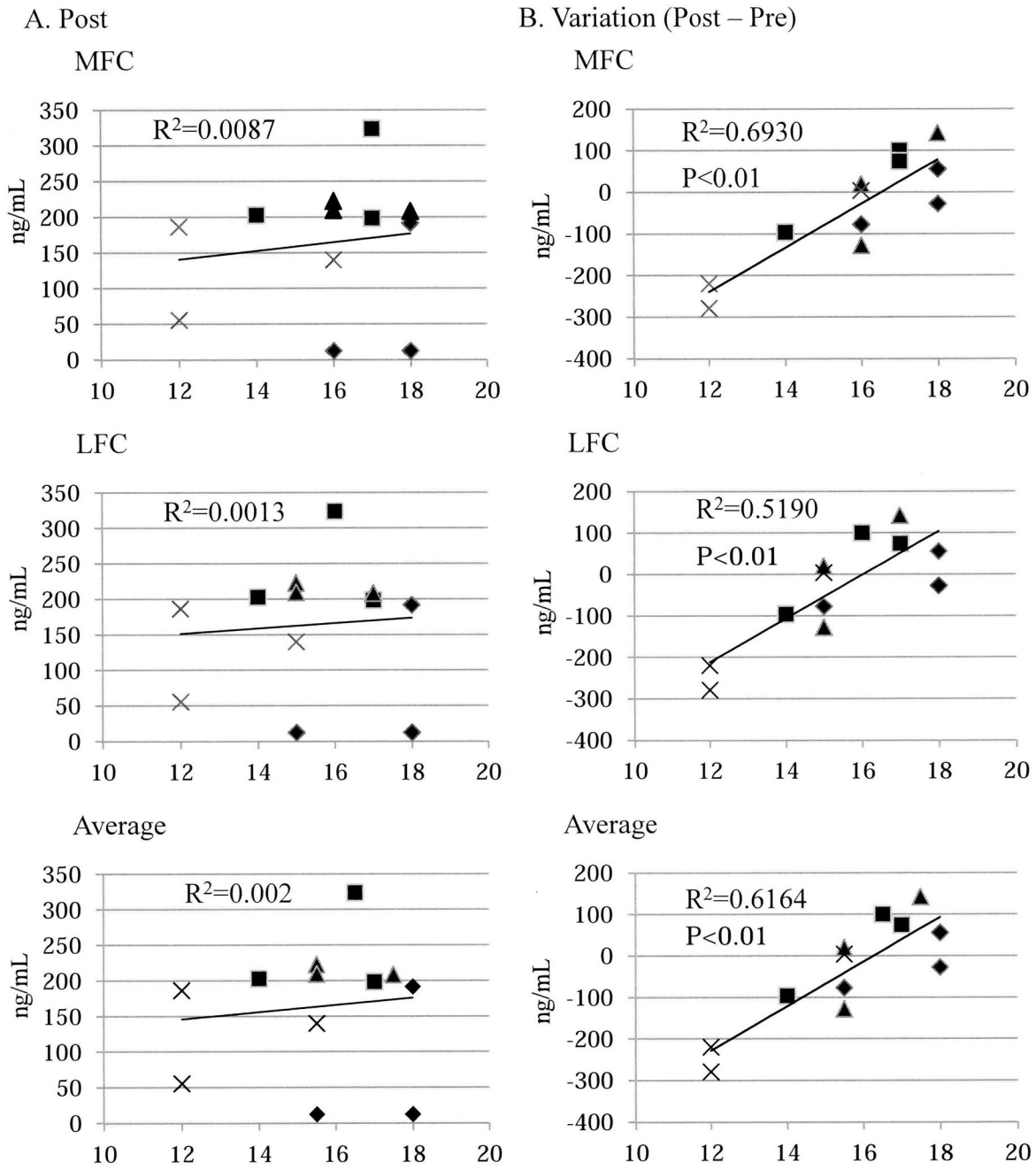

Fig. 6. Correlation of histological score and serum CS 846 concentrations

The scatter plot diagrams show the correlation between the histological score and CS 846 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 changes 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.

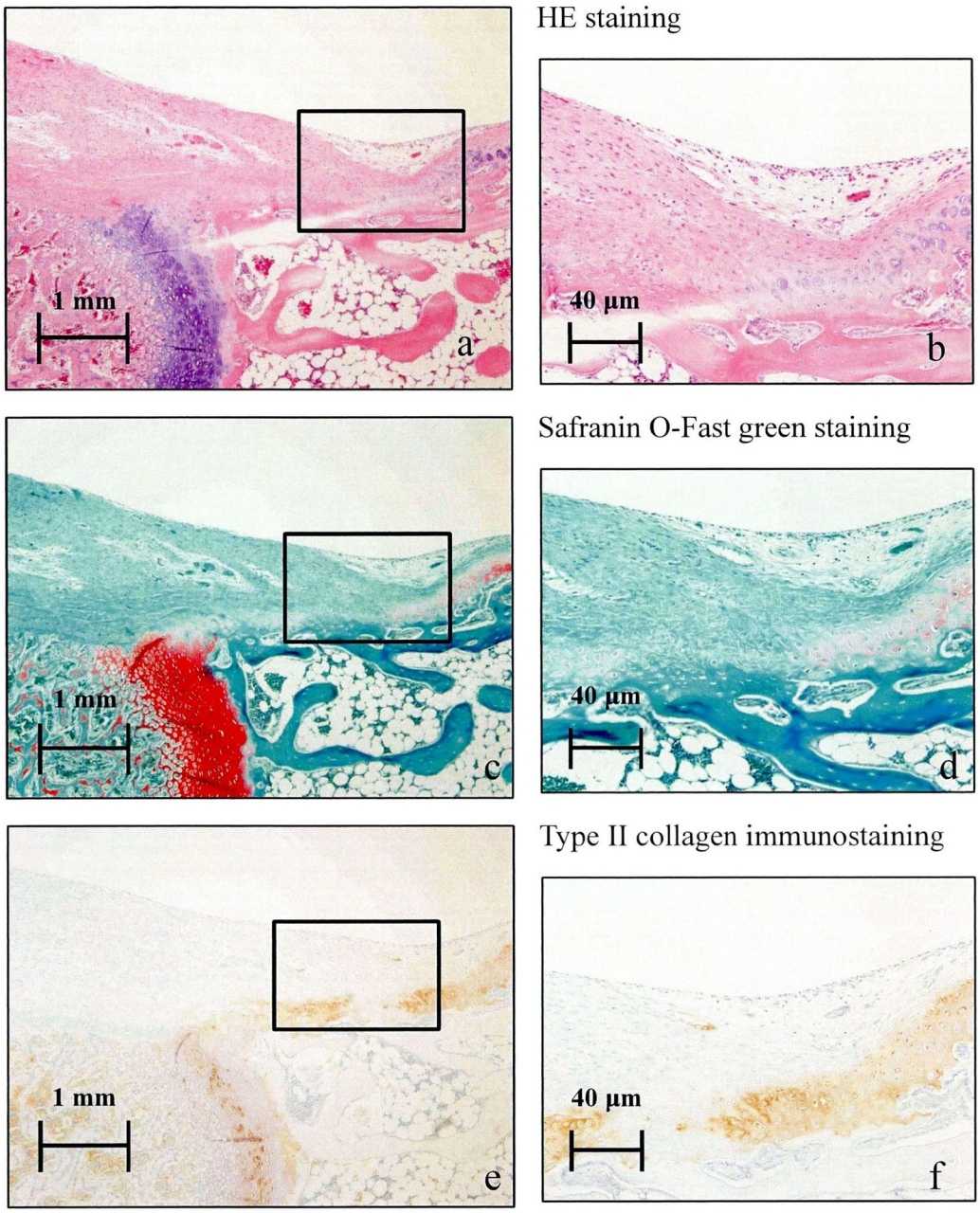

Fig 7. Histological findings of the lateral femoral condyle in C group at 4 week post-surgery

a: Low-magnification finding; The cleft extends into the middle zone.

b: High-magnification findings of the inset in Fig. 1a, in which the surface was covered by fibrous tissue.

c: Low magnification finding; There is a loss of red Safranin O staining in all 3 zones.

d: High magnification findings of the inset in Fig. 1c. The red staining Safranin O around the cells is not seen.

e: Low magnification of type II collagen immunostaining, in which the surface is

covered by fibrous instead of cartilaginous tissue. Collagen staining is only shown in deep zone.

f: High magnification of the inset in Fig. 1e. Staining of type II collagen is only seen in deep zone.

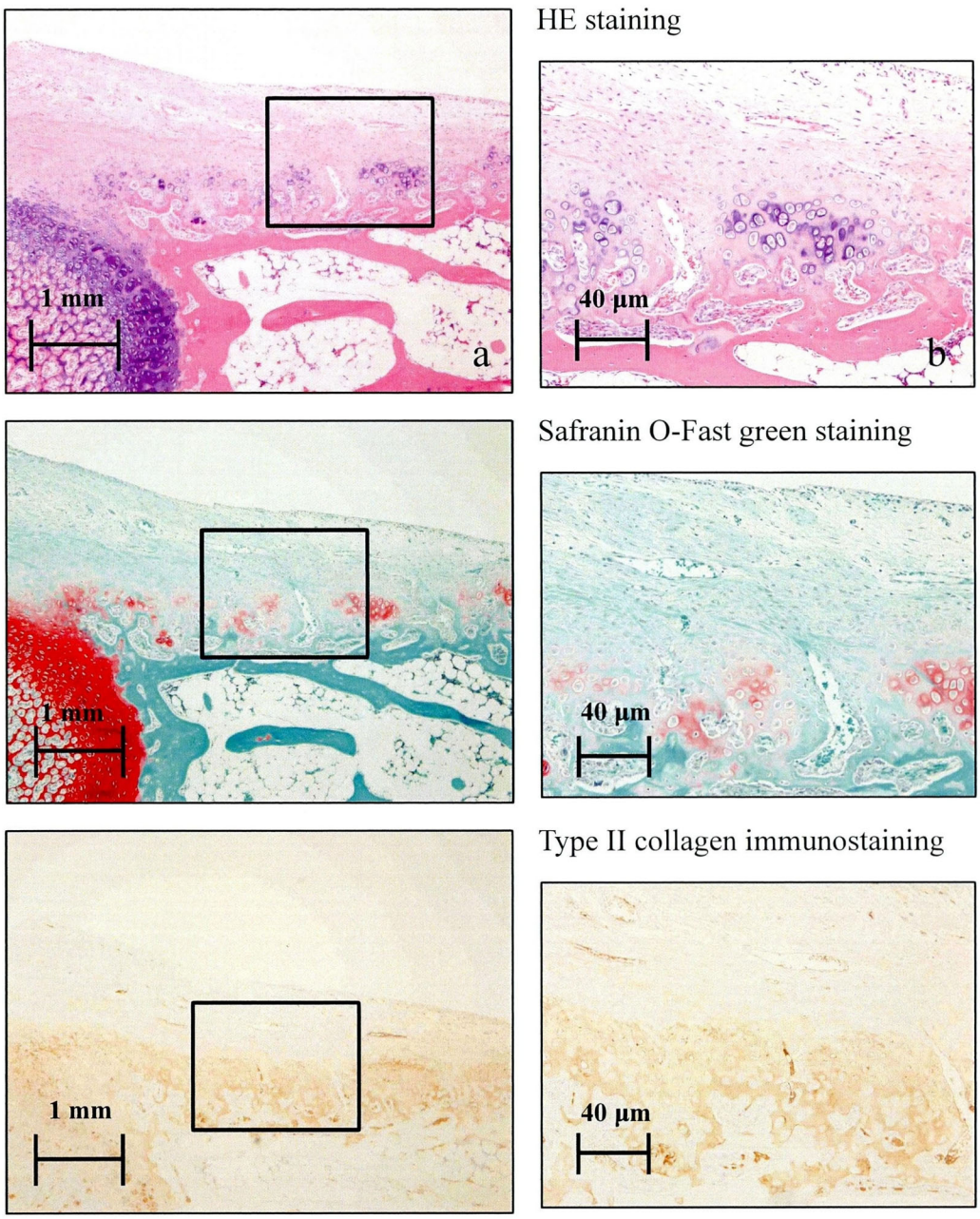

Fig 8. Histological findings of the lateral femoral condyle in FG group at 4 week post-surgery

a: Low-magnification findings; The cleft extends to the middle zone, but chondrocytes were shown in deep zone.

b: High-magnification findings of the inset in Fig. 1a, in which there are chondrocytes in deep zone.

c: Low magnification findings; the surfaces shows only irregularities , but Safranin O staining is still seen in the deep zone.

d: High magnification of the inset in Fig. 2c. There are chondrocytes and red staining

around the cells in the deep zone.

e: Low magnification of type II collagen immunostaining, in which the surface is covered by fibrous instead of cartilaginous tissue. The staining remain sin the deep zone.

f: High magnification of the inset in Fig. 3e showing staining of type II collagen around the cells in the deep zone.

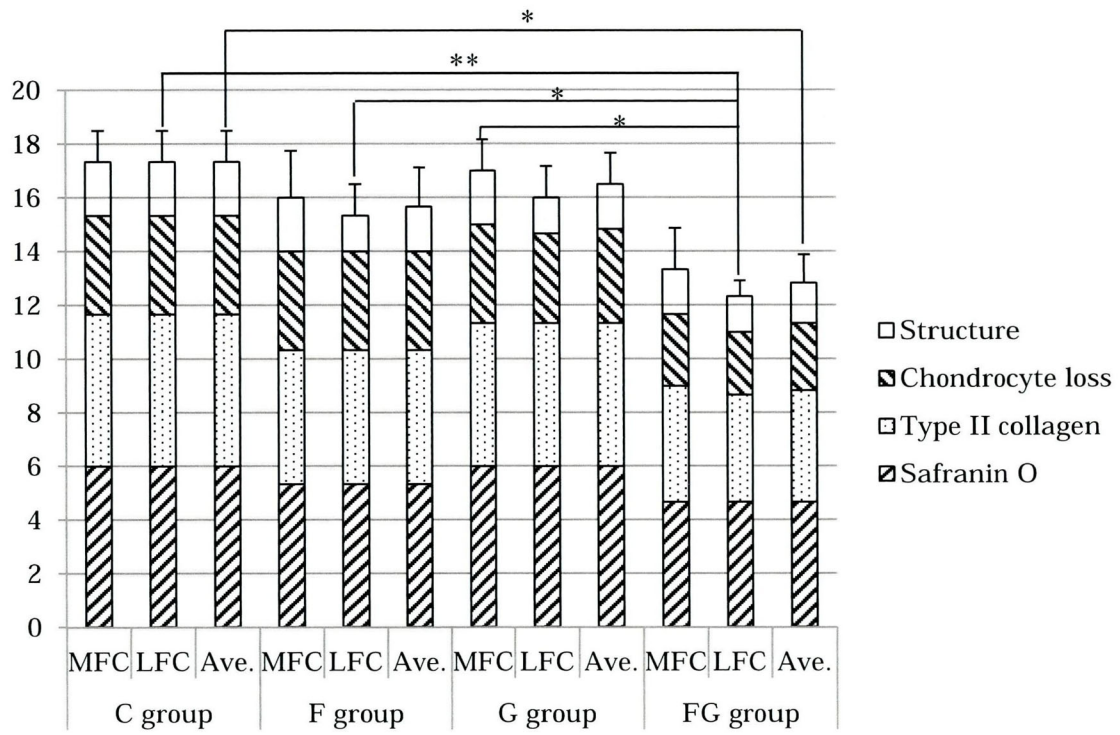

Fig 9. Histological assessment scores of each site (medial femoral condyle [MFC], lateral femoral condyle [LFC], and average).
Values are presented as means ± SD. (n = 3). * $P < 0.05$, ** $P<0.01$

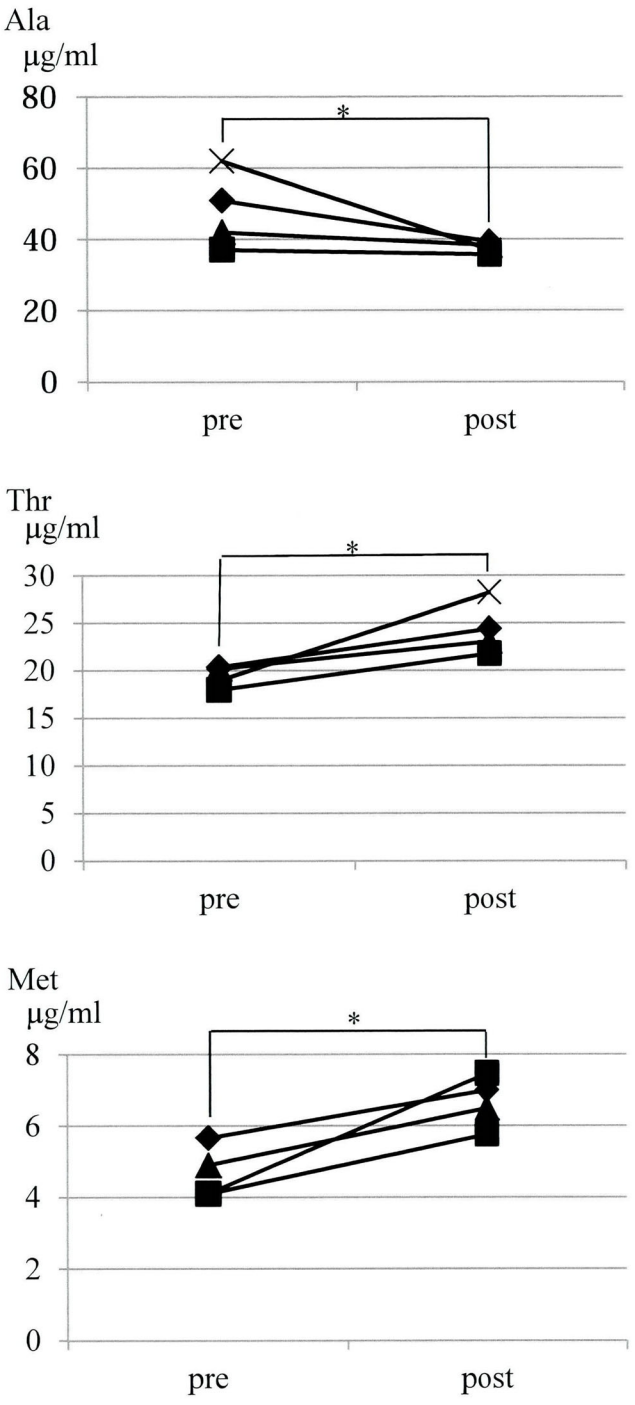

Fig 10. Comparison between pre- and post-ACLT in each amino acids

These change of the amino acids' concentration between pre- and post-ACLT showed significant differences in these amino acids. Values are represented as means of each group. Statistical analyses compared the whole post with pre and performed it. *: $P<0.05$

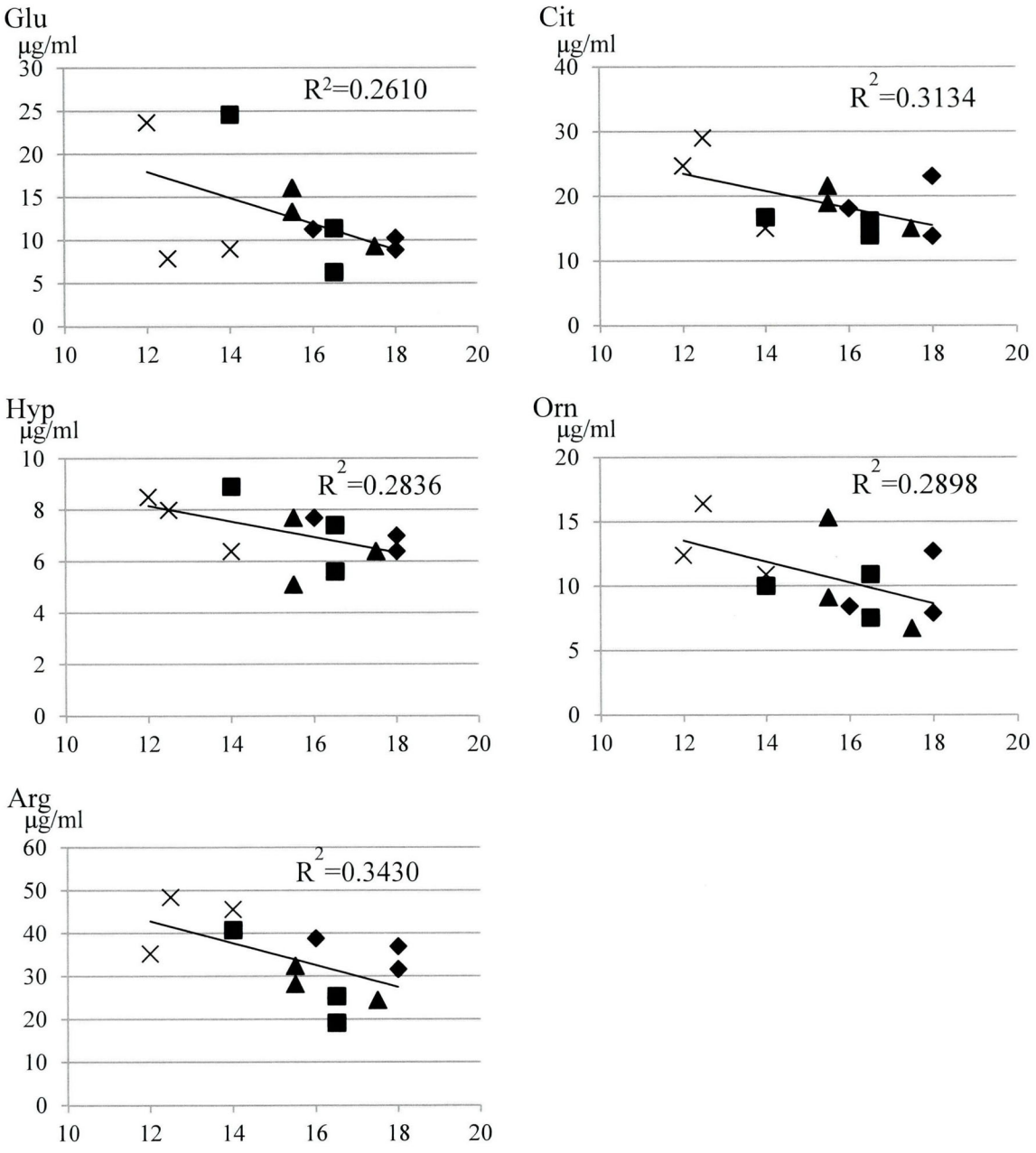

Fig 11. Correlation between histological score and plasma amino acids' concentrations at post-ACLT

Scatter plot diagrams showing the correlation between histological score at average and amino acids' concentrations at post-ACLT. The x-axis shows the histological score. The slope in the scatter plot represents the regression line. Arg was shown the significant correlation with histological score ($P<0.05$), and Glu, Hyp, Cit, Orn were shown the tendency to correlate with histological score ($005<P<0.10$).