EFFECT ON EXPERIMENTAL DAMAGED CARTILAGE REPAIR BY N-ACETYL-D-GLUCOSAMINE AND FISH COLLAGEN PEPTIDE

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Abstract

We investigated the synergistic wound healing effects of N-acetyl-D-glucosamine (GlcNAc) and fish collagen peptide (MW: 3,000 Daltons, water soluble collagen) on experimentary injured cartilage in rabbits. Under general anesthesia, three holes at articular cartilage of medial trochlear (one hole) and trochlear sulcus (two holes) of distal femur were created using high-speed orthopedic drill. After surgery, rabbits were orally administered with fish collagen peptide (FCP group, 1.2 g/head/day), and simultaneous administration of FCP (1.2 g/head/day) and GlcNAc (1 g/head/day), or for 2 weeks (FG group). Control group was provided with water ad lib. Each group used 3 rabbits. After 2 weeks, the holes were filled by fibroblasts and chondroblasts in the treatment groups, whereas in the control group, the holes were not filled completely like in the treatment groups. In the FG group, the holes were filled by much chondroblasts besides remodeling of bony trabeculae than the FCP groups. Histological image analysis of Safranin O and Alcian blue stained specimens revealed increasing of proteoglycans and glycosaminoglycans in the injured site in the treated groups. Most enhanced healing of injury and increase in Safranin O and Alcian blue positive materials was observed in the FG group. As a result, simultaneous administration of FCP and GlcNAc gave a fairly enhanced healing on restoration of cartilage injuries.

Introduction

Cartilage, a quite important joint support system for human and also animals, is a highly and specific differential tissue without having vasculature, lymphatic and nervous systems. In highly developed vertebrate, joint cartilage existed in a surface of epiphysis acts as weight supporting and motional point is mainly constructed by hyaline cartilage. Cartilage tissue is mainly consisted by highly developed extra cellular matrix produced by chondrocytes which are contained only 2~3 % in cartilage. An extra cellular matrix contains 70~80 % of water, 20~25 % of collagen, and 5~10 % of proteoglycans (PGs) [1]. In an extra cellular matrix, collagen fibers (mainly types II, VI, IX and XI) made dense network structure and will act as tension for maintain a form of cartilage. PGs aggregate to form agrrecan, and create expanding osmotic pressure by absorbing of water into collagen fiber network [2]. An aggrecan exists as compressed state in collagen fiber network. The state creates an expand pressure in a cartilage, and the pressure will act as bearing a compressed weight bearing with a minimum deformation in a cartilage tissue [3]. In general, it is well agreeable for fate of cartilage that chondrocytes maintain matrix synthesis by nutritious supplementation from joint capsule, however ability of matrix synthesis is gradually decreasing with aging. Poor ability of matrix synthesis causes a degenerative joint disease (DJD), especially in human stifle joint. Over athletics also induce DJD by mechanical abrasion to the joint [4].

In the present, treatment of DJD is employed medical application such as oral administration of non-steroidal anti-inflammatory drugs (NSAIDs), steroids, glucosamine, chondroitin supplements

with athletic rehabilitation or surgical treatments such as hyarulonic acid intra-joint injection, autograft of chondrocytes or stem cells implantation, or joint replacement [5], [6]. Glucosamine has a weak anti-inflammatory reaction compare to NSAIDs however, it is suitable for long time administration because of low toxicity and no side effects [7], [8]. From 1980, glucosamine was widely used to DJD patient in Europe countries for pain control [9]. However, in vivo experimental data have not been shown. We had investigated the effects of glucosamine (GlcN) on experimentally created cartilage damage using rabbit model on 2002 [10]. We also investigated the effects of N-acetyl-D-glucosamine (GlcNAc), Glucronic acid (GlcU) and glucose using same methods [11]. From the results of these experiments, glucose did not show the effect on cartilage regeneration, but GlcN, GlcNAc and GlcU showed chondroblasts regeneration with PG synthesis. Furthermore, we also investigated the effects of poultry collagen peptide and its synergistic effects on the cartilage regeneration with glucosamine. The collagen peptide also had the effect on cartilage repair, and synergistic effects were clearly observed. In the present paper, we investigated the effects of cartilage regeneration by fish collagen peptide and its synergistic effects with GlcNAc.

Material and Methods

Experimental animals

Twelve clinically healthy rabbits (Japanese Albino, females with the average age of 12 weeks) with a body weight of 2.5~3.0 kg were used. The experimental animals were used in the experiment after 1 week of acclimatization to the laboratory environment.

Experimental drugs: Water-soluble fish collagen (FCP): Collagen is extracted from fish skin, and is degraded by proteinase to various sizes of peptide (Yaizu Suisankagaku Industry, CO., Shizuoka, Japan). The mean molecular weights of FCP prepared are 3, 000. The major amino acids of FCP are glycine (24.6% of dry matter), glutamic acid (10.8%), proline (10.6%), and alanine (9.5%), respectively.

N-acetyl-D-glucosamine

Chitin obtained from crabs changed into oligomer through hydrochloric acid treatment and then the resultants were degraded into monomer by enzyme degradation (Yaizu Suisankagaku Industry, Co., Ltd., Shizuoka, Japan) was used. Purification of GlcNAc is 100 %.

Experimental grouping for drugs

The experimental rabbits (N=9) were divided into three groups: namely, control (C group), fish collagen peptide (FCP group), FCP and GlcNAc (FG group), and number of rabbit in each group is three.

Oral administration of GlcNAc and FCP

The control group had tap water to drink freely. Also, rabbits in the other groups were able to drink the tap water after ensuring that the daily dosage of each agent was administered. For the FCP group, 1.2 g of powdered FCP/head/day was dissolved into a half dosage of daily drinking water for each rabbit, and administered. After confirming completely drinking of each drug by each rabbit, and then the rabbit had tap water freely. The FG group had 1.2 g of FCP and 1 g of GlcNAc every day. Experimental duration is 2 weeks for each group.

Preparation of the model of articular cartilage injury

An experimental cartilage injury was made by drilling described by Tamai et al. (2002) [10]. In briefly, under general anesthesia, approaching from the lateral portion of the knee joint, an incision was made vertically on the skin from the central part of the femur toward the tibial tuberosity. The articular capsule was incised and the patella of the stifle joint was exposed completely by artificially dislocating the patella toward the medial side. Three holes of 2 mm size in diameter and 4 mm in depth were made by a hand-drill (Micro-engine D-2, Osada medical, Tokyo) at the articular cartilage of medial trochlear and trochlear sulcus (two holes) of distal femur. During the one-week period after the operation, the wound surface was disinfected by povidone-iodine (Isodine, Meiji confectionery, Tokyo) once a day and 10 mg/kg of Oxytetracycline (Terramycin, Pfaizer, Tokyo)

was subcutaneously administered twice a day to prevent infection. We named the created injured holes as follows: the holes in the trochlear sulcus describes as proximal and distal, and one in the medial trochlear as medial.

Post-mortem examination

At 3 weeks post-operation, the rabbits were euthanized by overdose (80 mg/kg) intravenous injection of pentobarbital (Nembutal, Dainippon Pharmaceutical Co., Osaka). The stifle joints were opened and were macroscopically observed at the operated site for determination of the synovial fluid contents and the injured cartilage.

Evaluation of the healing at injured sites

The degrees of restoration of the defective pores were classified into the following categories. Less than 50%: -; less than 60%: +; less than 80%: ++; 100% completely restored: +++. In addition, for statistical processing purposes, the above evaluation was assigned numbers: (-) was assigned with 0 point, (+) was assigned with 6 points, (++) was assigned with 8 points and (+++) was assigned with 10 points. The total value for each part was calculated. Quantified values were statistically processed by a Mann-Whitney's test at the p-level of 0.05 and below being considered to be statistically significant.

Histological observation

The recovered left femur was fixed by a 10% neutral buffered formaldehyde solution. After decalcification by 5% formic acid solution, the tissue was soaked for neutralization in the 5% sodium sulfate solution for a day, and then was washed for approximately 10 hrs under running water. After applying the usual method of embedding paraffin, the tissue was sliced by a microtome into 5 micron slices. Staining was carried out using the hematoxylin/eosin double staining method.
Histological repair ratio was measured by microscopic observation of hematoxylin/eosin staining specimen. Histological repair ratio (HRR) is depth of hole from non tactile cartilage surface at 2 weeks/original hole depth (4 mm) x 100.

Image analysis on proteoglycans (PG) and glycosaminoglycan (GAG)

Carrying out safranin O stains with the purpose of staining proteoglycans, and Alcian blue stains with the purpose of staining GAG, we observed the difference between restored substances at the injured parts in all groups. The 200 times magnified images of restored parts, articular cartilage and growing zone stained with safranin O stains and Alcian blue stains were taken into the computer by Photo grab ab-300 version 1.0 (Macintosh software, Fuji film, Tokyo) and the images digitized by using Adobe Photoshop 3.0 (Macintosh software, Adobe System, Tokyo). Then, the proportion of the pixel number that is accounted for by the desired hue in the total of 120,000 pixels (random sampling of 20,000 pixels at 6 locations) was calculated through the image processing technique. The obtained values were tested by a statistical processing method (t-test) and were considered statistically significant at the p-level of 0.05 and below.

Results and Discussion

Change in the body weight

All groups showed an increase in weight postoperatively. There was no animal in all groups where abnormal change was observed in their general condition.

Macroscopic findings (Figure 1)

In the control, there is no obvious change in the surgically created holes especially in the medial and the proximal holes, whereas, the other groups, the holes were buried with regenerated tissue, and the FG group showed best recovery than the FCP group.
In the control, there are no cases with holes buried over 80% of depth with regenerated tissue. In the proximal and the distal holes, the FG groups showed over 80% in 2 out of 3 cases, and in all cases, respectively. There is significant difference in total scoring points between the control group (total scoring points = 4+/-0.3) and the FCP group (7.3+/-0.3, $p<0.004$) and the FG groups (8.7+/-0.3,

p<0.001). The total scoring points of FG group is significantly higher than that of the FCP group (p<0.005).

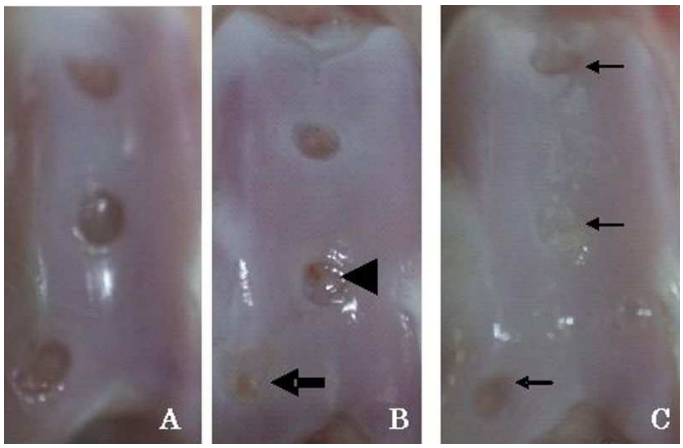

Figure 1 Macroscopic findings of the experimentary damaged cartilage at 2 weeks post operation.
A: the control group showed no obvious change in the surgically created holes.
B: the FCP group showed created holes buried by regenerated tissue, especially in the distal (arrow head) and the medial (arrow)
C: the FG group showed the three holes almost buried by regenerated tissue (arrows).

Histological findings

In the control (Figure 2), tissue defect was still clearly observed. The surface of wound was covered with regenerated connective tissue, and at deeper zone, a lack of bone trabecula and migration of macrophages, neutrophils and lymphocytes were observed. Saflanin O and Alcian blue positive materials were not found in the wounded area. In the FCP group (Figure 3), mature chondrocytes were massively regenerated in the wounded area, and also regenerated immature chondroblasts producing matrix surrounding the mature chondrocytes. Repairing of bone trabecula also observed under the regenerated chondrocytes. Saflanin O and Alcian blue positive materials were clearly observed around the regenerated chondrocytes. In the FG group (Figure 4), massive regenerated mature chondrocytes took possession of almost wounded area except for surface area. Immature chondroblasts were also observed in the deeper site of the wound. In the regenerated cartilage tissue, Saflanin O and Alcian blue positive materials were strongly observed. In our previous paper, showed also chondrocytes regeneration. The level of chondrocytes regeneration in the GlcNAc group was much higher than that of the FCP group, but lower than that of the FG group.

HRR of the control was significantly lower than the other group. HRR of FG group is also significantly higher than the GlcNAc and FCP groups.

Results of image analysis on safranin O and Alcian blue stained specimens

In safranin O staining, the FG group showed significant increase of positive materials than the control. In Alcian blue staining, the FCP and the FG groups had significant difference than the control. However, there is no significant difference on positive materials in both staining analysis between the FCP and the FG groups.

Cartilage regeneration

From the present results, the FCP has a cartilage regenerative effect on experimentally damaged cartilage, and also more effective on simultaneous administration with GlcNAc. Subchondral trabecular bone repair also observed in the three groups except for the control, and its effect was superior in the FG group.

In general, ability of self repair of articular cartilage has been known as quite low, so once damage happen in it; the damaged areas are repaired by covering of fibrous tissue or fibrous cartilage. However, these regenerated tissues, even in fibrous cartilage do not have function as articular

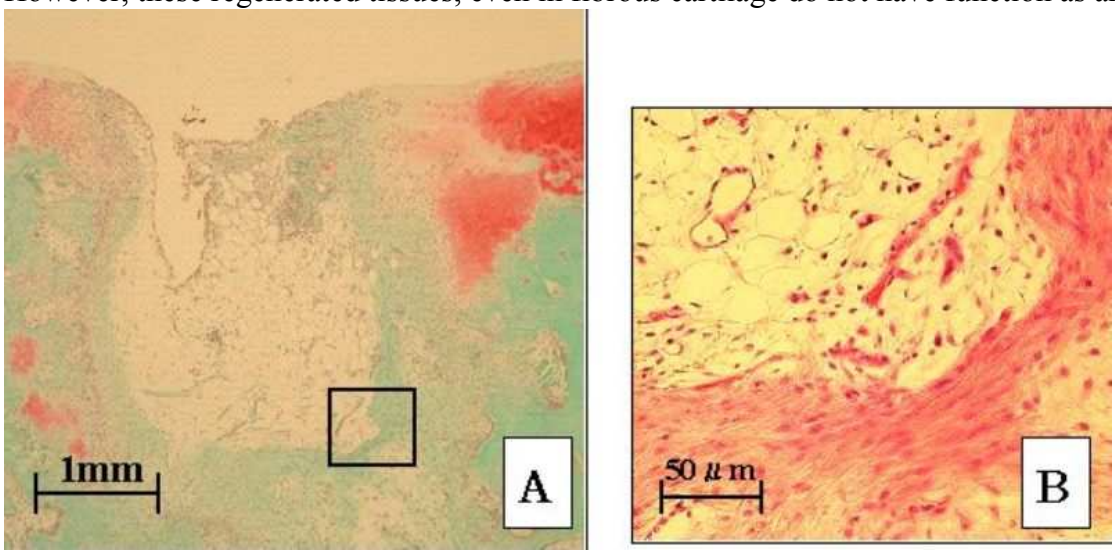

Figure 2 Histological findings of the control at 2 weeks (Saflanin O staining).
A: Lower magnification of damaged area. No Saflanin O positive materials regenerated in the wounded area.
B: Hyper magnification of square area in the photo A (HE staining). The regenerated tissue was only connective tissue consisted by fibroblasts and fatty tissue with few vasculature.

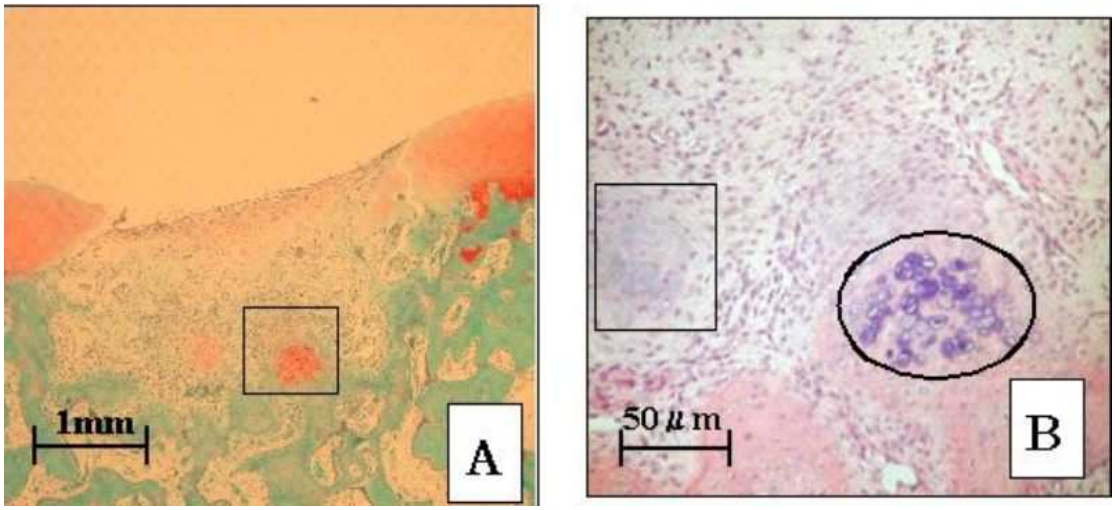

Figure 3 Microscopic findings of the FCP group at 2 weeks post operation.
A: Safranin O staining of the specimen. Safranin O positive materials regenerated in the wounded area. Subchondral bone also repaired.
B: Hyper magnification of the square area in the A (HE staining). Mature cartilage tissue regenerated (the round area) with surrounded by immature chondroblasts regeneration.

cartilage [12]. In the present experiment, regenerated cartilage tissue was hyaline cartilage confirming by special staining for glycosaminoglycan (GAG) and PGs.
Shapiro et al. (1993) [13] demonstrated cartilage repair mechanism using autoradiography, full thickness damage of articular cartilage including subchondral bone was repair by immature mesenchymal cells derived from bone marrow. The mesenchymal cells migrated from marrow produce type I collagen and little extracellular matrix during initial differentiation [12]. Finally the cells differentiated to chondrocytes produce type II collagen and PGs. We already reported full

thickness defects of articular cartilage model in a rabbit [10], [11], [14], and no cartilage regeneration in the defect site within 3 weeks after surgery in the control wound. The wounded hole was covered by only fibrous connective tissue and no cartilaginous tissue regenerate even flattened

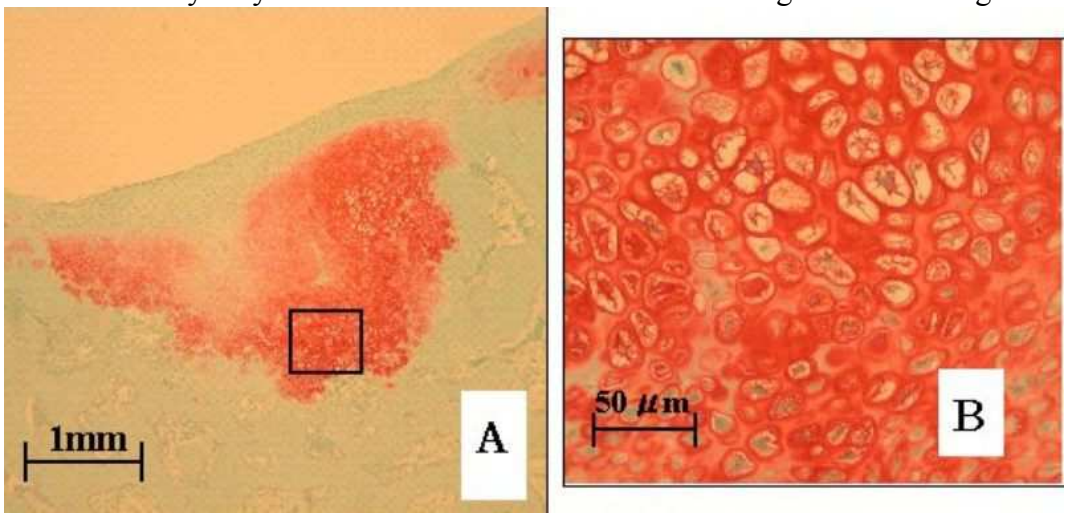

Figure 4 Microscopic observation of the FG group at 2 weeks post operation.
A: Saflanin O staining specimen of wounded area. Mach positive materials regenerated and almost filling the hole at wounded area Subchondral bone also regenerated.
B: Hyper magnification of the square area in A. Mature chondrocytes regenerated with proteoglycans synthesis (saflanin O staining).

fibrocartilaginous cells. Shapiro et al. (1993) [13] reported in the same experimental report of rabbit model, the differentiation of mesenchymal cells begin around 10 days and become flattened fibrocartilaginous cells at two weeks, and finally they observed well demarcated layer of cartilage containing chondrocytes at 3 weeks post surgery. The deference between our data and the Shapiro's data [13] would be a deference of keeping method of rabbits. They keep rabbits moving freely, and we keep them in an individual cage. In the present data and our previous GlcN, GlcNAc and poultry collagen peptide data, cartilage tissue was regenerated at 2 weeks post operation. FCP and GlcNAc combination administration accelerate cartilage regeneration almost one week compare to the Shapiro's data [13] and more two weeks compare to our present and the previous control data.
Cartilage matrix is mainly consisted with collagen and PGs. In the present study, orally administered FCP and GlcNAc would contribute synthesis of the both substances. Simple FCP administration induces significant increase of GAG synthesis in the regenerated tissue, therefore, some amino acids such as glutamic acid, one of the rich amino acids in FCP accelerate GAG synthesis by activation of L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) and catalyze synthesis of glucosamine-6-phosphate [15]. Furthermore, in a process of GAG synthesis by chondrocytes, GlcNAc will act as basic material. GlcNAc stimulates GAG syntheses in chondrocytes and prevents degeneration of GAG in vitro [16]. Shikman et al. (2001) [17] also reported GlcNAc suppressed production of cyclooxygenase-2 (COX-2) and IL-6 from chondrocytes by suppression of NO production from normal human chondrocytes, and control of IL-1 and TNF α activation on chondrocytes. From our present histological results and other previous investigations, FCP and GlcNAc simultaneous or single oral administration causes increasing of PG and GAG synthesis in regenerating tissue in the wounded area.
Good subchondral bone repair also observed in the both groups except for the control. Shapiro et al. (1993) [13] and Wakitani et al. (1994) [18] reported in the experimental full thickness defect in rabbits, subchondral bone plate was repair at 24 weeks after operation. On the other hand, Wang & Hu (2004) [19] reported in their experimental auto graft of chondrocytes in collagen gel in rabbit model, hyaline cartilage was regenerated in 4 weeks and the repair of subchondral bone was 12 weeks. The present result showed excellent wound healing acceleration by FCP and GlcNAc. About

a mechanism of regeneration, mesenchymal cells derived from marrow differentiate to chondroblasts and osteoblasts [13], [18], and a mechanism of acceleration of wound healing, Wang & Hu(2004) [19] and Buma et al. (2003) [20] pointed out type I and II collagen will be supportive activation for differentiation of mesenchymal cells.
In comparison with our previous data (GlcN [10], GlcNAc [11], GlcU [11], and simultaneous administration of GlcN and poultry collagen peptide [14]), simultaneous administration of FCP and GlcNAc showed more accelerating wound healing in the wounded area. It will be needed further investigation including not only total amino acid variation especially in the variation of amino acids concerning to collagen synthesis but also a mechanism of differentiation of mesenchymal cells to chondrocytes.

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