WOUND HEALING ACCELERATION OF EXPERIMENTAL DAMAGED TENDON BY GLCNAC AND FISH COLLAGEN PEPTIDE.

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Abstract

We investigated the restorative effects of orally administered fish collagen peptide (FCP) and N-acetyl-D-glucosamine (GlcNAc) on the experimentally produced tendon injuries in rabbits. A microscopic tendon injury model was made by a total of 20 times penetration by 23G needle in the Achilles tendon. Sixteen rabbits were used in this experiment, and were divided into 4 groups. For the control group, only tap water was administered daily and for the FCP and GlcNAc groups, a water based solution of FCP (2.4g/head/day) and GlcNAc (1g/head/day) was administered daily, respectively. For the FCP/GlcNAc group, both samples were administered simultaneously. The condition of the injured part was observed macroscopically and histologically at 2 weeks after injury. Macroscopically, extensive thickness and neovasculature were observed in all cases in the control and GlcNAc groups. In the FCP group, in 2 out of 4 cases, extensive thickness and neovasculature were observed. In the FCP/GlcNAc group, in 2 out of 4 cases, extensive thickness was observed, while there was no neovasculature. Microscopically, many big scars were observed in the control group. A few small scar formation was observed in the FCP group. There was slight infiltration of neutrophils in the GlcNAc group. In the FCP/GlcNAc group, the injured sites were considerably restored. Image analysis was performed using histological photographs. Percentages of nuclear area in the field of view in the untreated, control, FCP, GlcNAc, and FCP/GlcNAc groups were 1.99%、16.99%、9.21%、12.37%、8.78%, respectively. The FCP and FCP/GlcNAc groups showed lowly significant value compared to that of the control group.

Introduction

We reported that D-glucosamine (GlcN), which is a basic component of chitin and chitosan, accelerated wound healing of the injured joint cartilage (Tamai, *et al*., 2002). We also reported that glucuronic acid and N-acetyl-D-glucosamine (GlcNAc), which are important key materials in biological synthesis glycosaminoglycans, accelerated wound healing of the injured joint cartilage, but glucose did not (Tamai, *et al*., 2003). For synthesis of proteoglycan, core protein synthesis is necessary. Furthermore, type II collagen that is a basic structure of cartilage must be regenerated. From this point, we investigated effect of collagen peptide on wound healing of injured cartilage, and found that oral administration of collagen peptide was effective to wound healing of the injured cartilage and its efficiency was increased by simultaneous administration of GlcN (Hashida, *et al*., 2003).

Tendon and ligament are specialized connective tissues that link muscle to bone and bone to bone. These tissues are characterized by poor vasculature, poor cell components, and abundant extra-

cellular matrix (Amadio, 1992). Once these tissues are injured, restore period is required for longer (Lin, *et al*., 2004). There is only conservative treatment and no active therapy. We hypothesized that amino sugar and collagen peptide have potency to enhance wound healing of tendon/ligament injury as well as cartilage, because histological characteristics of tendon/ligament resemble that of cartilage.

In the present study, we investigated effect of oral administration of GlcNAc and fish collagen peptide (FCP) on wound healing of Achilles tendon using a new tendon injury model.

Material and Methods

Animals: Sixteen clinically healthy rabbits (Japanese albino, female with a body weight ranging from 1.85-2.85 kg) were used. All rabbits were used in the experiment subsequent to the period of habituation for one week after the delivery.

Reagents: FCP and GlcNAc were provided from Yaizu Suisankagaku Industry (Japan). FCP was made by enzymatic degradation of collagen extracted from fish skin, and its average molecular weight was 3,000. GlcNAc was made by enzymatic degradation of chitin derived from crab shell, and its purity was 100%.

Experimental design: Under the general anesthesia with ketamine-HCl(25mg/kg IM, Ketalar injection, Sankyo, Japan) and medetomidine-HCl (0.1 mg/kg SC, Domitor, Meiji Confectionary, Japan), a surgical preparation ranging from left stifle joint to knee joint was performed. Skin incision of 1.5-cm above Achilles tendon was made. After exposure of Achilles tendon, 23G needle was penetrated a part of Achilles tendon (1-cm width from 1cm apart from attachment of the tendon to heel bone) at 20 times. Using suture material, skin incision was closed with a synthetic absorbent thread (USP 3-0 suture Opepolix, Nescosuture, Japan). Immediately after the operation, the action of medetomidine-HCl was antagonized by atipamezole-HCl (0.5 mg/kg IM, Antisedan, Meiji Confectionary, Japan). During 3 days after operation, oxytetoracycline hydrochloride (10 mg/kg SC, Terramycin, Pfizer, USA) was administered once a day as an antibiotic to prevent infection.

The animals were divided into 4 groups (n=4), FCP, GlcNAc, FCP/GlcNAc, and control groups at random. In the control group, the animals were given only tap water to drink freely. For the FCP and GlcNAc groups, a water based solution of FCP (2.4g/head/day) and GlcNAc (1g/head/day) was administered daily, respectively. For the FCP/GlcNAc group, both samples were administered simultaneously.

Macroscopic observation: Diarrhea, appetite, coat color, and body weight were observed during experimental period. At 2 weeks post operation, the animals were euthanized by an overdose (80 mg/kg) intravenous injection of pentbarbital sodium (Nembutal, Dainippon Pharmaceutical Co., Japan). The Achilles tendons were exposed and were macroscopically observed about neovasculature and thickness at the injured sites.

Microscopic observation: The Achilles tendons were taken after macroscopic observation, and then fixed by a 10% neutral buffered formaldehyde water solution. After applying the usual method of embedding paraffin, the tissue was sliced by a microtome into 5 micron slices. Staining was carried out using hematoxylin/eosin double staining method. Under the 400 magnification, 10 fields at the injured sites in each section were observed at random, and then each field was scored based on criteria as follows. 1 point: inflammatory cells and fibroblasts are observed at the injured site; 2 point: immature collagen fibers are observed at the injured site, and inflammatory cells are less than that of 1 point; 3 point: fibroblasts mature and tenocytes with oval nuclei appear, and collagen fibers thicken, but alignment of the fibers is random; 4 point: collagen fibers thicken more, and alignment of the fiber is regular; 5 point: collagen fiber mature and resemble to normal tendon tissue.

Image analysis: Ten photographs of the injured site were taken at random under the 400 times magnification field using digital camera (Nikon digital camera E995, Nikon, Japan), and the images were digitized by digital microscope (VHX2000, KEYENCE Co., Japan) (Figure 1). Then, the proportion of cell nucleus area in the total field of view was calculated.

Statistical analysis: The obtained values were tested by Fisher's test and were considered statistically significant at the *p*-level below 0.05.

Results and Discussion

Macroscopic finding: During experimental period, there was no abnormal finding in general condition such as diarrhea, vomiting, and weight loss in all animals. Table 1 shows macroscopic finding at the injured site. In the control and GlcNAc groups, extensive thickness and neovasculature were observed in all cases. In the FCP group, in 2 out of 4 cases, extensive thickness and neovasculature were observed. In the FCP/GlcNAc group, in 2 out of 4 cases, extensive thickness was observed, while there was no neovasculature.

Microscopic findings: Many big scars were observed in the control group (Fig. 2A). In the FCP group, many immature tenocytes observed at the injured sites, and alignment of collagen fibers was random. A few smaller scar formation was observed. In the GlcNAc group, alignment of collagen fibers was regular and slight inflammatory cells were observed. In the FCP/GlcNAc groups, the injured sites were considerably restored (Fig. 2B). Figure 3 shows histological score of each group. Score of the GlcNAc group was significantly more than that of the control group. In the FCP and FCP/GlcNAc groups, there was no significant difference compared to the control group.

Image analysis: Figure 4 shows percentage of nuclear area in the field of view. The FCP and FCP/GlcNAc groups showed lowly significant value compared to that of the control group.

Discussion

From the present results, oral administration of FCP and GlcNAc found to accelerate wound healing of the tendon as well as cartilage. Furthermore, it was found that simultaneous administration of FCP and GlcNAc was most effective.

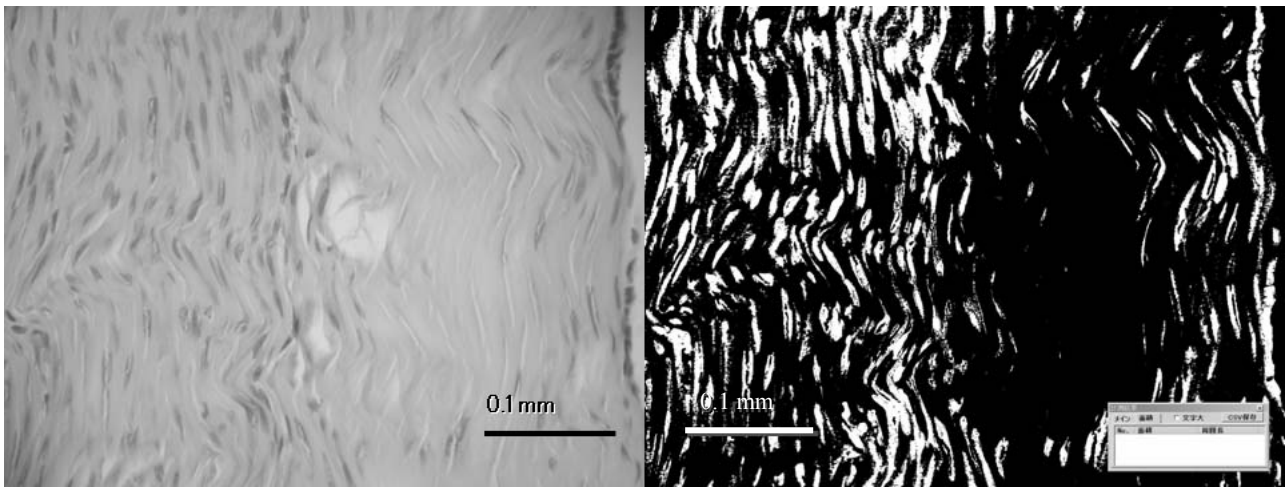
In general, protein is degraded to peptides and amino acids in intestine, and absorbed from intestinal mucosa. Peptides are degraded to amino acids in intestinal mucosa. After that, amino acids are used for protein re-synthesis and activation as co-enzyme for synthesis of another amino acids *in vivo*. It is well known that in a medication of amino acids, volume and balance are important. If a certain amino acid is ingested excessively, ingestion of another amino acids is inhibited. In collagen that comprises a large percentage of tendon tissue, many glycine, proline, and hydroxyproline are contained. Therefore, it is important that these amino acids are absorbed effectively for restoration of tendon. In the FCP, many amino acids are contained in a balanced manner. The FCP contains glycine, glutamine acid, arginine, proline, hydroxyproline, asparagine acids, and serine more than 5%, compared to beef. We noted metabolisms of these amino acids *in vivo*. Glycine is metabolized to pyruvic acid through serine, and pyruvic acid goes into TCA cycle as acetyl CoA. Glutamine acid is metabolized to alpha-ketoglutamine acid, which goes to TCA cycle. Asparagine acid goes to TCA cycle as oxaloacetic acid, and is related to synthesize acetyl CoA and citric acid, which circulates TCA cycle through alpha-ketoglutamine acid. Arginine is hydrolysed by arginase, and become to urea and ornithine. Ornithine is related to synthesize proline. Proline has 2 pathways. One pathway is that proline is related to synthesize glutamine acid and goes to TCA cycle. Another pathway is that proline is hydrated by prolylhydroxylase and become to hydroxyproline. Hydroxyproline is degraded to glyoxylic acid and pyruvic acid. In the FCP, many glycine, proline, hydroxyproline, and serine are contained. Therefore, it is thought that almost these amino acids are used as an energy production through TCA cycle, but that a part of these amino acids are stocked *in vivo*. We speculate that these stocked amino acids are used effectively to collagen synthesis, subsequently accelerate wound healing of tendon.

In the present study, it was observed that inflammatory phase was accelerated by GlcNAc administration. Minagawa *et al.* (2006) reported that GlcNAc induced type I matrix metalloproteinase (MMP-1) *in vivo*. Okamura *et al.* (2005) reported that fibroblasts induced MMP-1 in the presence of GlcNAc *in vitro*. MMP-1, -2, -3, and –13 are recognized as a collagenase. MMP-1 resolves type I collagen, MMP-2 resolves collagen fiber and induces resolution and degeneration of type I collagen (Tsuzaki, *et al*., 2003). MMP-3 is produced by tenocytes and acts as well as MMP-1. MMP-13 is also produced by tenocytes and degrades collagen (Tsuzaki, *et al*., 2003). In the injured tendon, type I collagen is decreased and type III collagen is increased in early phase. After that, type I collagen is increased again and restoration of tendon is achieved. In the present study, it is thought that MMP-1 induced by GlcNAc promoted degradation and resolution of type I collagen at the injured site, and subsequently made transfer quickly from inflammatory phase to remodeling phase in wound healing process. Tenocytes have IL-1 receptor. When tendon is injured, macrophages and monocytes infiltrate at the injured site and release IL-1. The IL-1 released induces COX-2 and MMPs in the injured site (Tsuzaki, *et al*., 2003; Yang, *et al*., 2005). In addition to histological evaluation, it is necessary to evaluate wound healing of tendon using cytokine measurement in future.

Table 1. Summary of macroscopic findings

Group	Thickness	Neovasculature
Control	4/4*	4/4
FCP	2/4	2/4
GlcNAc	4/4	4/4
FCP/GlcNAc	2/4	0/4

*Number of observed / total number



Before image processing **After image processing**

Figure 1. Image processing. Nucleus as blue ranging from 0 to 128-colored was changed to white in color, and another colors were to black in color. The percentage of white area in the field of view was calculated.

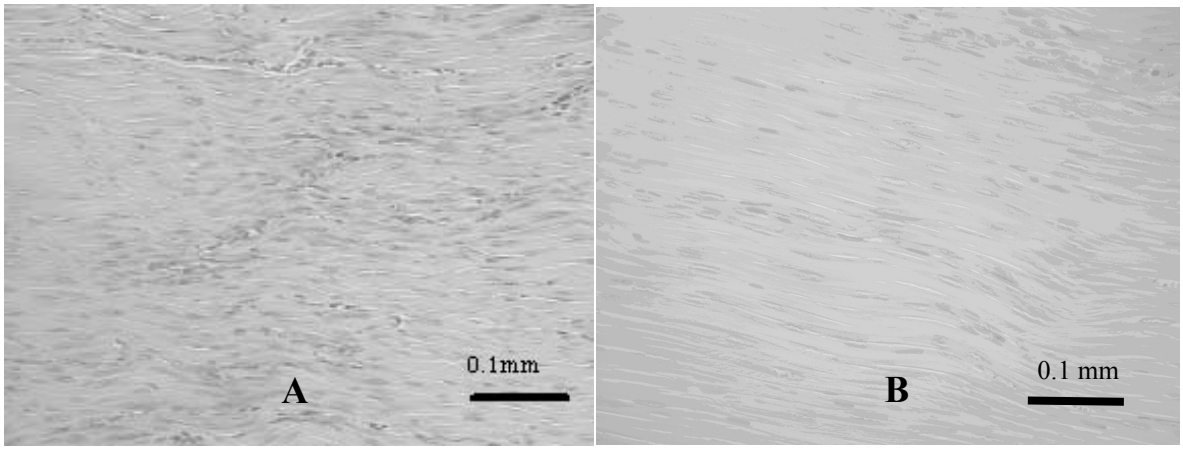


Figure 2: A: Control group. Many big scars were observed. B: FCP/GlcNAc group. The injured sites were considerably restored.

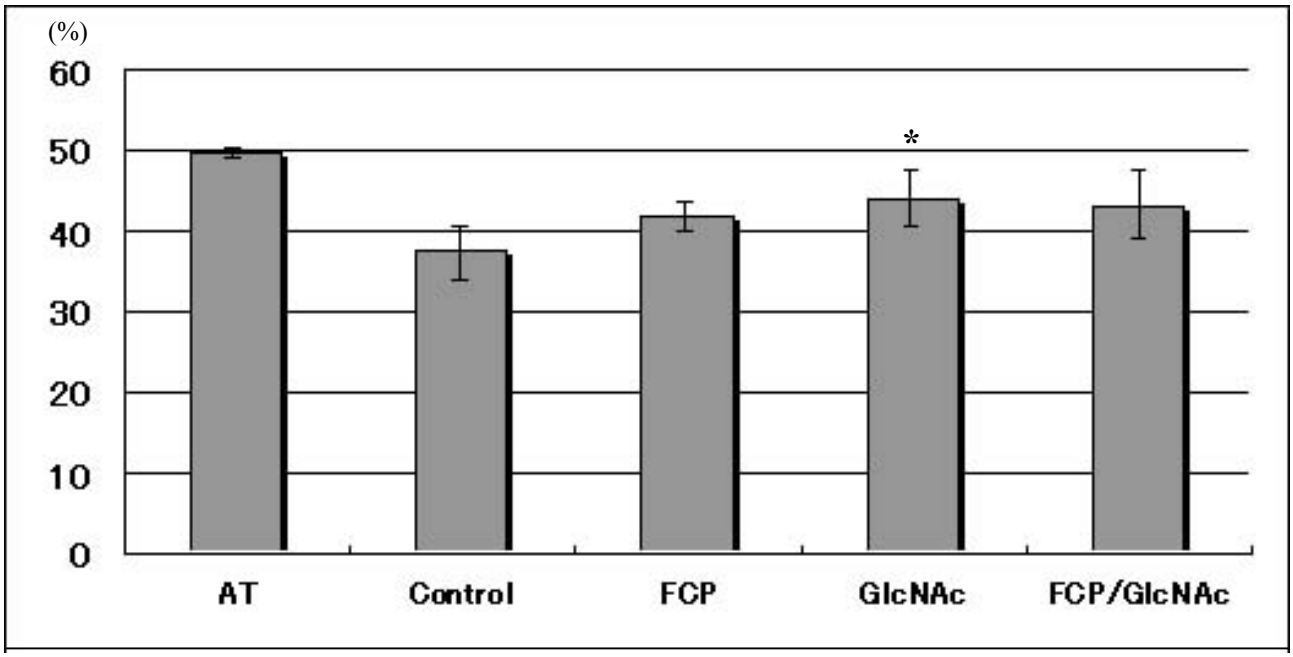


Figure 3: Histological score of each group. AT is atraumatic tendon, which means no injured tendon.

***: p<0.05 vs. control**

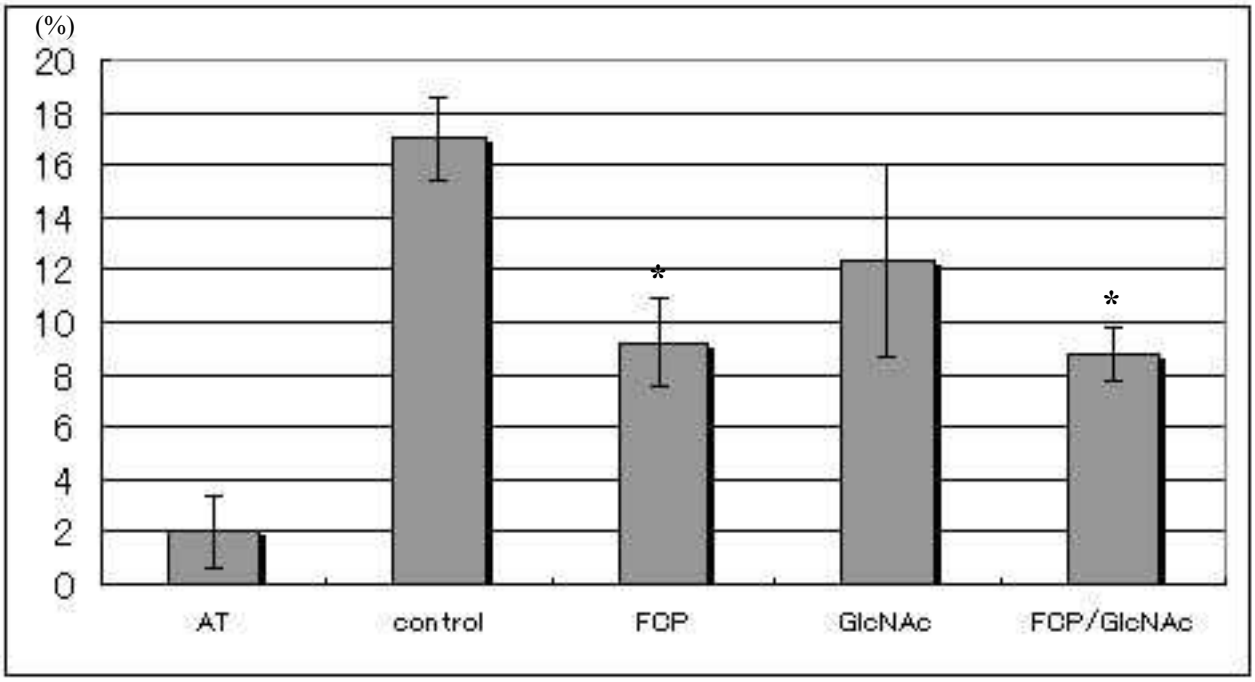


Figure 4: Perecentage of nuclear area in the field of view. AT is atraumatic tendon, which means no injured tendon. *: p<0.05 vs. control

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