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Enhanced healing of cartilaginous injuries by *N*-acetyl-D-glucosamine and glucuronic acid

Yasunori Tamai[a], Katsuyuki Miyatake[a], Yoshiharu Okamoto[a], Yoshimori Takamori[b], Koji Sakamoto[b], Saburo Minami[a,*]

[a]*Department of Veterinary Surgery, Faculty of Agriculture, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan*
[b]*Koyo Chemical Co., Ltd, 2-1-11 Koraku, Bunkyo-ku, Tokyo 112-0004, Japan*

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Abstract

We investigated the restorative effect of orally administered glucose, *N*-acetyl-D-glucosamine (GlcNAc) and glucuronic acid (GlcUA) on the experimentally produced cartilaginous injuries in rabbits. A total of three holes in the left stifle joint, including one in the medial trochlear ridge, and two in the trochlear sulcus (proximal and distal) of articular cartilage were made surgically using a drill. For the control group, only tap water was administered daily and for the glucose, GlcNAc, GlcUA groups, a water based solution (1 g/head/day) of glucose, GlcNAc, glucuronolactone was administered daily, respectively. We observed the clinical symptoms daily and the condition of the injured part was observed macroscopically and histologically at 3 weeks after the operation. There was no difference in body weight or general conditions among each group. With respect to medial trochlear injury, 1/3 holes were not cured in the control, but all were cured in the glucose, GlcNAc and GlcUA groups, respectively. With respect to the proximal hole, 4/6 in the control group, 3/3 in the glucose and 2/3 in the GlcNAc were not cured. However, 2/3 in the GlcUA were cured. There was significant difference ($p < 0.05$) in the proximal holes between the control and the GlcUA. On the total points, there was significant difference ($p < 0.05$) between the control and GlcNAc or GlcUA.

On histological examination, the injured parts were covered by fibrous connective tissues in the control and the glucose, whereas in the GlcNAc and GlcUA groups, the massive proliferation of matured cartilaginous tissues was observed, and the regenerated cartilaginous tissues were surrounded by the proliferation of chondroblast cells. In the regenerated tissue, matured cartilage substrate was also observed. Safranin O and Alcian blue stains marked a more significantly dense in the GlcNAc and GlcUA group than in the control ($p < 0.01$) in injured parts as well as in non-injured joint cartilage.

Keywords: *N*-acetyl-D-glucosamine; Glucuronic acid; Articular cartilage

1. Introduction

Recently, efficacy of various nutritional products, called slow-acting disease-modifying osteoarthritis agents, has been drawing attention in treatment and prevention of osteoarthritis in humans and animals. Cartilage is an important connective tissue in the body and it is a highly differentiated organ that lacks blood vessels, lymph system and nerves. Among high vertebrate animals, cartilage exists particularly on the surface of the weight bearing part of the joint. Having only 2–3% of the volume of the joint, the cartilage cells are composed mostly of highly organized extracellular matrix. Cartilaginous matrix consists of approximately 70–80% of water content, 20–25% of collagen, 5–10% of proteoglycan (PG) (Maroudas, Bayliss, & Venn, 1980; Muir, 1973). Collagen fiber (mainly types II, VI, IX and XI) generates a dense mesh structure and PG (mainly aglycan) that forms the shape of matrix and generates tension characteristics which has a function of generating expansive pressure by absorbing water in the tissues through osmotic pressure (Yoshihara & Shiina, 1994). Mesh structures of collagen do not have potential extensive force, therefore, the compressed aglycan in it plays quite important role to generate expansive pressure of it. This creates an ideal tissue that can stand a compressive load with minimal deformation, and supports the function of the cartilage surface to support load with sturdiness and elasticity (Hardingham & Fosang, 1992). PG that performs this

* Corresponding author. Tel./fax: +81-857-31-5433.
E-mail address: minami@muses.tottori-u.ac.jp (S. Minami).

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important role is composed of glycosaminoglycan (GAG) and protein, while GAG is in turn composed mainly of extracellular matrix polymers such as hyaluronic acid, chondroitin sulfate, keratan sulfate, heparan, heparin sulfate and dermatan sulfate. As a constituent of the polymer chains, D-glucosamine or *N*-acetyl-D-glucosamine (GlcNAc) is drawing attention as an important key element (Theodosaikis et al., 1997). The glucose, source of body kinetics of PG and GAG, is main energy source in body. GlcNAc is a monosaccharide product generated from chitin by hydrolysis and is categorized into hexosamine. On the other hand, glucuronic acid (GlcUA) is one of GAG component except for keratan sulfate. GlcUA has been used for treatment of liver disease, however, no report for osteoarthritis treatment. GlcUA is also converted from glucose, and is used for GAG synthesis. The ability of PG synthesis in the body declines with aging. With aging, incapacitates of PG generation is gradually progressing, and it is known that the incapacitation results in senile osteoarthritis (McDevitt & Muir, 1976). There are no reports concerned with GlcUA for osteoarthritis treatments or regeneration and healing of joint cartilage. We have already reported the efficacy of glucosamine hydrochloride (GlcN) on healing cartilage injuries (Tamai et al., 2002). In the present study, we investigated other key materials such as glucose, GlcNAc and GlcUA on effects of cartilage regeneration.

2. Materials and method

2.1. Animals

Fifteen clinically healthy rabbits (Japanese albino, 3 males, 12 females with the average age of about 12 weeks) with a body weight of approximately 2.0 kg were used. Six rabbits were allocated to the control. In the glucose, the GlcNAc and the glucuronolactone three rabbits were allocated, respectively. All rabbits were used in the experiment subsequent to the period of habituation for 1 week after delivery.

2.2. Reagents

D-Glucose, molecular weight 180.16, was purchased from Wako Pure Chemical (Osaka). Glucuronolactone (Sumitomo Chemical Finechem Co., Ltd, Osaka) was used for origin of GlcUA. GlcNAc whose purity is 98.5% was obtained from Koyo Chemical (Tokyo).

2.3. Experimental design

General anesthesia was carried out with intramuscular injection of 25 mg/kg, ketamine–HCl (Ketalar injection, Sankyo, Tokyo) after sedation by subcutaneous injection of 0.1 mg/kg, medetomidine–HCl (Domittor, Meiji Confectionary, Tokyo). Rabbit hair at the left knee joint was clipped and after being disinfected with Chlorhexidine (Hibiscrub, Zeneca, Osaka, Japan) and 70% alcohol. 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 lateral side. Three holes of 2 mm size in diameter and 4 mm deep were made by a hand-drill (Micro-engine D-2, Osada Medical, Tokyo) at the articular cartilage of medial trochlear one hole and trochlear sulcus (two holes) of the distal femur. Afterwards, the incision wound made during the operation was rinsed by sterile saline and the articular capsule was sutured with a synthetic absorbent thread (USP 3-0 suture PDS, Johnson & Johnson, USA). The subcutaneous tissues and the skin were sutured at the same time with nylon thread (USP 3-0 suture, Suprylon, Vomel, Germany). Immediately after the operation, the action of Medetomidine–HCl was antagonized by 0.5 mg/kg of atipamezole–HCl (Antisedan, Meiji Confectionary,

Table 1
Effect on body weight by glucose, glucuronic acid and *N*-acetyl-D-glucosamine administration

Experimental group	Sex	Body weight (kg)	
		Pre-operation	At the time of autopsy
Control group			
1	Female	2.1	2.6
2	Female	2.6	3.2
3	Female	2.6	3.3
4	Male	2.4	2.8
5	Male	2.3	2.8
6	Male	2.0	2.4
Mean		2.3	2.9
Standard deviation		0.3	0.3
Glucose group			
1	Female	2.1	2.7
2	Female	2.3	3.0
3	Female	2.1	2.8
Mean		2.2	2.8
Standard deviation		0.1	0.2
Glucuronic acid group			
1	Female	2.0	2.8
2	Female	2.2	2.7
3	Female	2.4	3.1
Mean		2.2	2.9
Standard deviation		0.2	0.2
N-acetyl-D-glucosamine group			
1	Female	2.1	2.8
2	Female	2.2	2.8
3	Female	2.1	2.7
Mean		2.1	2.8
Standard deviation		0.1	0.1

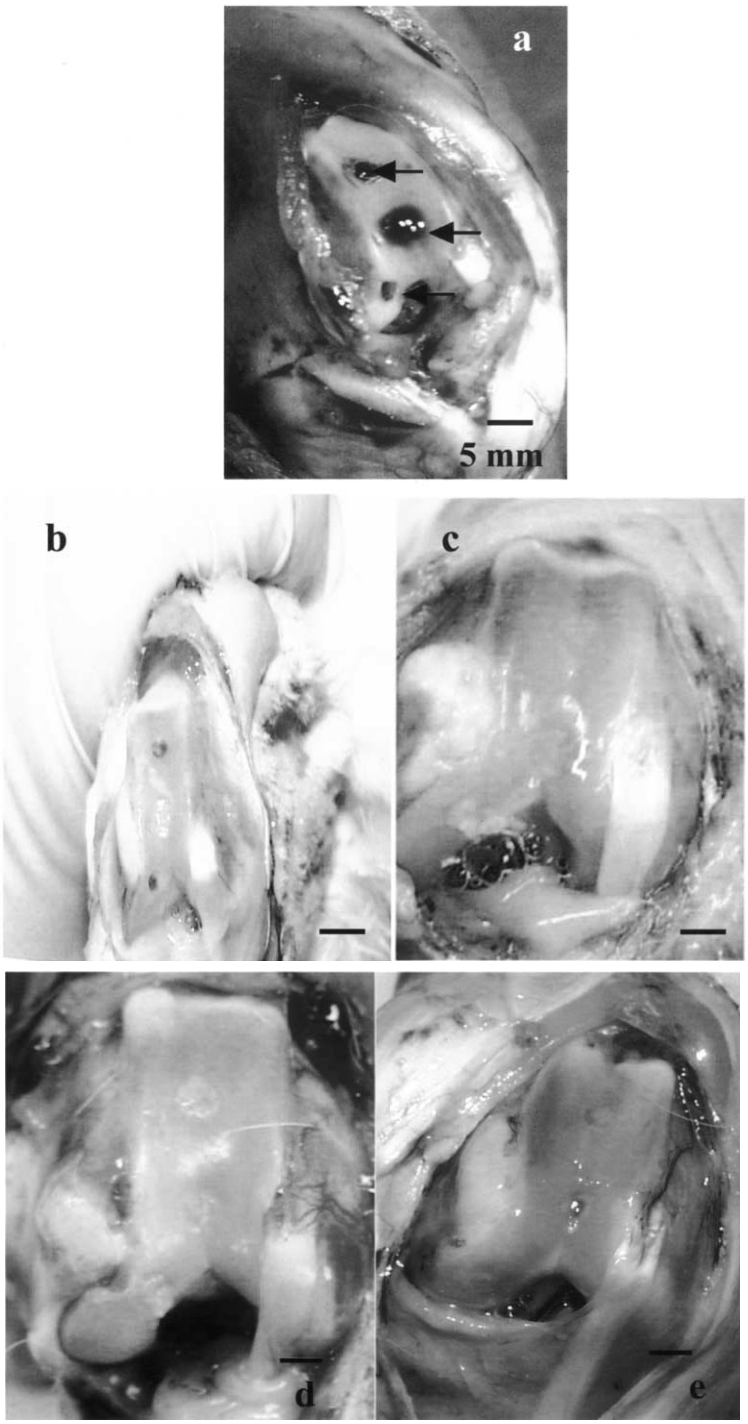

Fig. 1. Surgically created articular cartilage injuries and macroscopic findings of the operated sites at 3 weeks after the operation. (a) Three holes of the size 2 mm in diameter and 4 mm in depth were made by a hand-drill in the knee joint of the distal femur bone. From top to bottom, we called proximal hole (trochlear sulcus), distal hole (trochlear sulcus) and medial hole (medial trochlea). (b) The findings of rabbits in the control group at 3 weeks after the operation. It shows that the medial and the proximal are not healed. (c) The findings of rabbits in the *N*-acetyl-D-glucosamine group at 3 weeks after the operation. Three holes were perfectly healed. (d) The findings of rabbits in the glucuronic acid group at 3 weeks after the operation. Three holes were perfectly healed. (e) The findings of rabbits in the control group at 3 weeks after the operation. It shows that the proximal and distal trochlear sulcus is not healed.

Tokyo) intramuscularly. Tranexamic acid (Ranobis, Isei, Yamagata, Japan) was administered intravenously 20 mg/kg as a hemostatic drug as well as for speedy recovery consciousness. During the 1 week period after the operation, the wound surface was disinfected by povidone iodine (Isodine, Meiji confectionary, Tokyo) once a day and oxytetracycline hydrochloride 10 mg/kg (Terramycin, Pfizer, Tokyo) was subcutaneously administered twice a day as an antibiotic to prevent infection. We describe the surgically made holes as follows: the holes in the trochlear sulcus

Table 2
Macroscopic findings of the operated sites at 3 weeks after the operation

Experimental group	Sex	Degree of healing[a]		
		Medial trochlea ridge	Trochlear sulcus (proximal)	Trochlear sulcus (distal)
Control group				
1	Female	++	+	+++
2	Female	+++	+++	++
3	Female	+++	+	++
4	Male	+++	+	+
5	Male	+++	+	++
6	Male	+	+++	+++
Glucose group				
1	Female	+++	++	++
2	Female	+++	+	++
3	Female	+++	++	++
Glucuronic acid group				
1	Female	+++	++	+++
2	Female	+++	+++	+++
3	Female	+++	+++	++
N-acetyl-D-Glucosamine group				
1	Female	+++	+++	+++
2	Female	+++	++	+++
3	Female	+++	++	++

[a] −: less than 50% healing; +: 50–60% healing; ++: 60–80% healing; +++: 80–100% healing.

describe as proximal and distal, and the one in the medial trochlear as medial.

Six rabbits (3 males and 3 females) of the control group were given only tap water to drink freely. Each three rabbits of the glucose, the GlcNAc and the GlcUA groups had a solution of powdered glucose, GlcNAc and glucuronolactone, respectively, dissolved in tap water that was administered every day at a rate of 1 g/head/day during

Table 3
Macroscopic findings of the operated sites at 3 weeks after the operation

Experimental group	Sex	Degree of healing[a]			Total points
		Medial trochlea ridge	Trochlear sulcus (proximal)	Trochlear sulcus (distal)	
Control group					
1	Female	2	1	3	6
2	Female	3	3	2	8
3	Female	3	1	2	6
4	Male	3	1	1	5
5	Male	3	1	3	7
6	Male	1	3	3	7
Mean		2.5	1.7	2.3	6.5
Standard deviation		0.8	1.0	0.8	1.0
Glucose group					
1	Female	3	2	2	7
2	Female	3	1	2	6
3	Female	2	3	2	7
Mean		2.7	2.0	2.0	6.7
Standard deviation		0.6	1.0	0	1.2
p^*					0.23
Glucuronic group					
1	Female	3	2	3	8
2	Female	3	3	3	9
3	Female	3	3	2	8
Mean		3.0	2.7	2.7	8.3
Standard deviation		0	0.6	0.6	0.6
p^*					< 0.05

Table 3 (*continued*)

Experimental group	Sex	Degree of healing[a]			Total points
		Medial trochlea ridge	Trochlear sulcus (proximal)	Trochlear sulcus (distal)	
N-acetyl-D-glucosamine group					
1	Female	3	3	3	8
2	Female	3	2	3	8
3	Female	3	2	2	7
Mean		3.0	2.3	2.7	7.7
Standard deviation		0	0.6	0.6	1.2
p*					<0.05

*Statistical analysis was performed between control and glucose, glucuronic acid and *N*-acetyl-D-glucosamine groups on each operated area and total points. There is significant difference in the glucuronic acid and *N*-acetyl-D-glucosamine at the total points ($p < 0.05$).

[a] 0 point: less than 50% healing; 1 point: 50–60% healing; 2 points: 60–80% healing; 3 points: 80–100% healing.

experimental period. Also, rabbits in the glucose, the GlcNAc and the glucuronolactone groups were able to drink the tap water after ensuring that the daily dosage of each drug was administered.

2.4. Macroscopic observation

Diarrhea, appetite, coat color and body weight were observed during the experimental period. At 3 weeks post-operation, the rabbits were euthanized by an overdose (80 mg/kg) intravenous injection of pentobarbital sodium (Nembutal, Dainippon Pharmaceutical Co., Osaka, Japan). The stifle joints were opened and were macroscopically observed at the operated site for determination of the healing of the injured cartilage. The degree of restoration of the experimentally produced of holes at the distal femur was observed macroscopically. The degrees of restoration of the defective holes were classified into the following categories: less than 50%: (−); 50–60%: (+); 60–80%: (++); 80–100%: (+++). In addition, for testing statistical significance, the above evaluation was assigned numbers: (−) was assigned with 0 point, (+) was assigned with 1 point, (++) was assigned with 2 points and (+++) was assigned with 3 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 to be considered statistically significant.

2.5. Muscle weight

The left and right lateral great muscles and the biceps of the femur were separated and sampled after euthanasia, and their fresh weights were taken, and the muscle weight ratio (%) was calculated by comparing the operated side with the non-operated side. The weight ratio (%) was examined by a Student's t-test (at the p-level of <0.05 being to be considered statistically significant).

Table 4
Effect of glucose, glucuronic acid and *N*-acetyl-D-glucosamine on the muscle weights of the femur

Experimental group	Sex	Degree of healing	
		Lateral great muscle	Biceps of the femur muscle
Control group			
1	Female	95.4	96.7
2	Female	91.2	91.2
3	Female	93.7	95.8
4	Male	92.2	89.1
5	Male	94.5	99.1
6	Male	97.2	99.1
Mean		94.0	95.2
Standard deviation		2.2	4.2
Glucose group			
1	Female	95.8	91.7
2	Female	90.6	92.1
3	Female	96.9	95.7
Mean		94.4	93.2
Standard deviation		3.4	2.2
Glucuronic acid group			
1	Female	95.8	96.2
2	Female	99.5	100.6
3	Female	94.3	98.9
Mean		96.5	98.6
Standard deviation		2.7	2.2
N-acetyl-D-glucosamine group			
1	Female	91.4	95.0
2	Female	94.8	97.2
3	Female	97.5	95.6
Mean		94.6	95.9
Standard deviation		3.1	1.1

Muscle weight ratio was calculated by following formula. Muscle weight ratio = operate side muscle weight/non-operate side muscle weight. There is no significant difference at the muscle weight ratio of lateral great muscle and biceps of the femur muscle.

2.6. Microscopic observation

The recovered left femur was fixed by a 10% neutral buffered formaldehyde water solution. After the fixation, the operated stifle joint was trimmed to a thickness of 5 mm and decalcified for a day with shaking in 5% formic acid solution. After decalcification, the tissue was soaked for neutralization in the 5% sodium sulfate solution for a day, and then was washed for approximately 10 h under running water. After applying the usual method of embedding paraffin, the tissue was sliced by a microtome into 5 μm slices. Staining was carried out using the hematoxylin/eosin double staining method. By carrying out Safranin O stains with the purpose of staining PG, and Alcian blue stains at pH 1.0 (only sulfate) and 2.5 (sulfate and carboxyl) with the purpose of staining GAG, we observed the difference between restored substances at the injured parts between groups (Spicer, 1976).

2.7. Image analysis

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 Photograb ab-300 version 1.0 (Macintosh Software, Fujifilm, Tokyo) and the images digitized by 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 Student's *t*-test and were considered statistically significant at the *p*-level below 0.05.

3. Result and discussion

3.1. Macroscopic findings

Table 1 shows the change in body weight before and after the operation. Every group showed an increase in weight post-operatively. However, there was no remarkable increase in the body weight in the Glc, GlcUA and GlcNAc groups. In addition, there was no animal in respective groups where abnormal change was observed in their general condition. In this experiment, the weights in the Glc group were distributed from 2.1 to 3.0 kg. As a result of this, the amount of Glc administered to the animals ranged from 333 to 476 mg/kg. The weights in the GlcUA group were distributed from 2.1 to 3.0 kg. As a result of this, the amount of GlcUA administered to the animals ranged from 333 to 500 mg/kg. The weights in the GlcNAc group were distributed from 2.1 to 2.8 kg. As

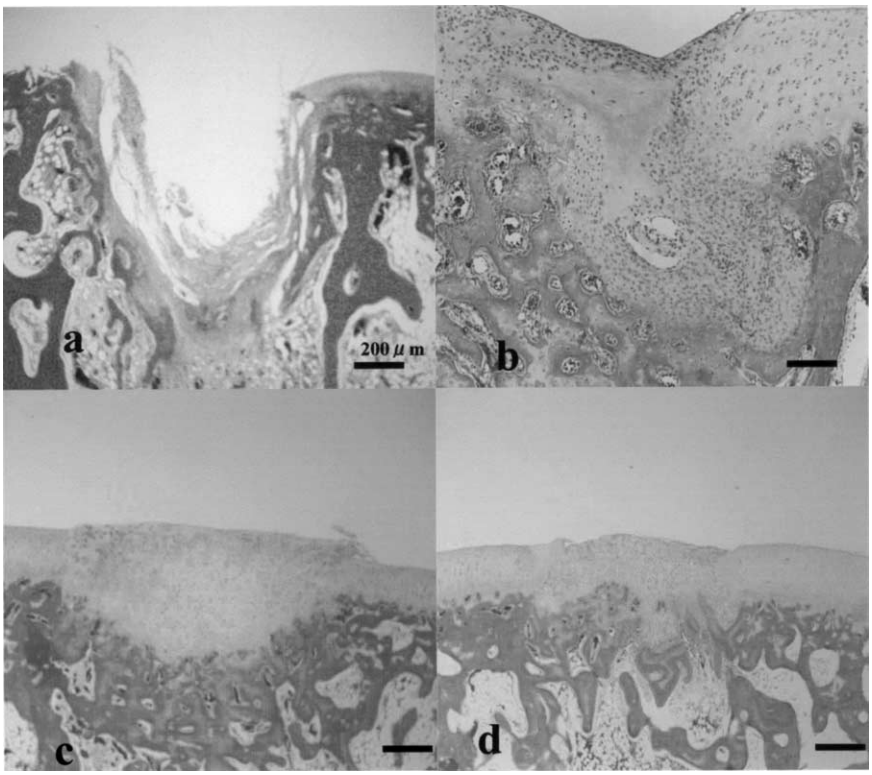

Fig. 2. Histological findings at 3 weeks after the operation (lower magnification images, HE staining). (a) In the control rabbits, the holes were covered by connective tissues and there was a slight regeneration of cartilage in the most medial surface. (b) In the glucose rabbits, the some regeneration of cartilage was observed, but much of restored part was hyaline and fibrous tissues. (c) In glucuronic acid rabbits, the regeneration of massive cartilage was observed and the restored of the bone crest was also observed. (d) In *N*-acetyl-D-glucosamine rabbits, the regeneration of massive cartilage was observed and the restored of the bone crest was already observed.

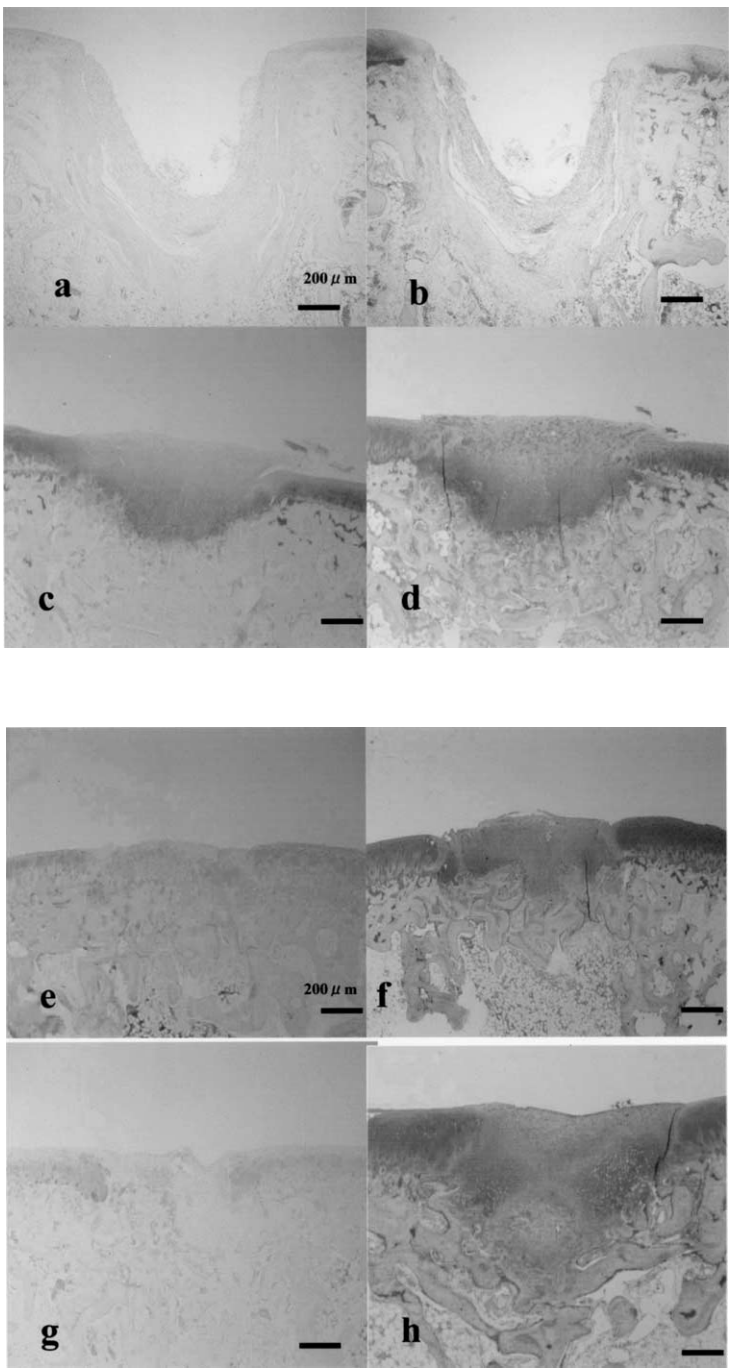

Fig. 3. Histological findings at 3 weeks after the operation (lower magnification images, Safranin O staining and Alcian blue staining). (a) Alcian blue staining image of rabbits in the control group of the same parts of the body. There is a small positive part in the injured parts. The degree of staining inside non-injured cartilage is poorer in comparison with rabbits in the glucosamine group. (b) In the Safranin O staining, the injured site of the control showed no positive Safranin O staining image. The strength of staining inside the cartilage of the non-injured parts is poor in comparison with it of the glucosamine group. (c) Alcian blue staining image of rabbits in the glucuronic acid group of the same parts of the body. There was a clear positive part at the injured parts inside the regenerated cartilage. The degree of staining inside the non-injured cartilage shows a clear increase in comparison with rabbits in the control group. (d) Safranin O staining image of rabbits in the glucuronic acid group of the same parts of the body. There was a clear positive Safranin O staining image at the injured parts inside the regenerated cartilage. The degree of staining inside the non-injured cartilage shows a clear increase in comparison with rabbits in the control group. (e) Alcian blue staining image of rabbits in the *N*-acetyl-D-glucosamine group of the same parts of the body. There was a clear positive part at the injured parts inside the regenerated cartilage. The upper part at injured part was deeply stained. (f) Safranin O staining image of rabbits in the glucosamine group of the same parts of the body. There was a clear positive Safranin O staining image at the injured parts inside the regenerated cartilage. The degree of staining inside the non-injured cartilage shows a clear increase in comparison with rabbits in the control group. (g) Alcian blue staining image of rabbits in the glucose group of the same parts of the body. There is a small positive part in the injured parts. The degree of staining inside non-injured cartilage is poorer in comparison with rabbits in the glucuronic acid and *N*-acetyl-D-glucosamine group. (h) In the Safranin O staining, the injured site of the glucose showed positive Safranin O staining image.

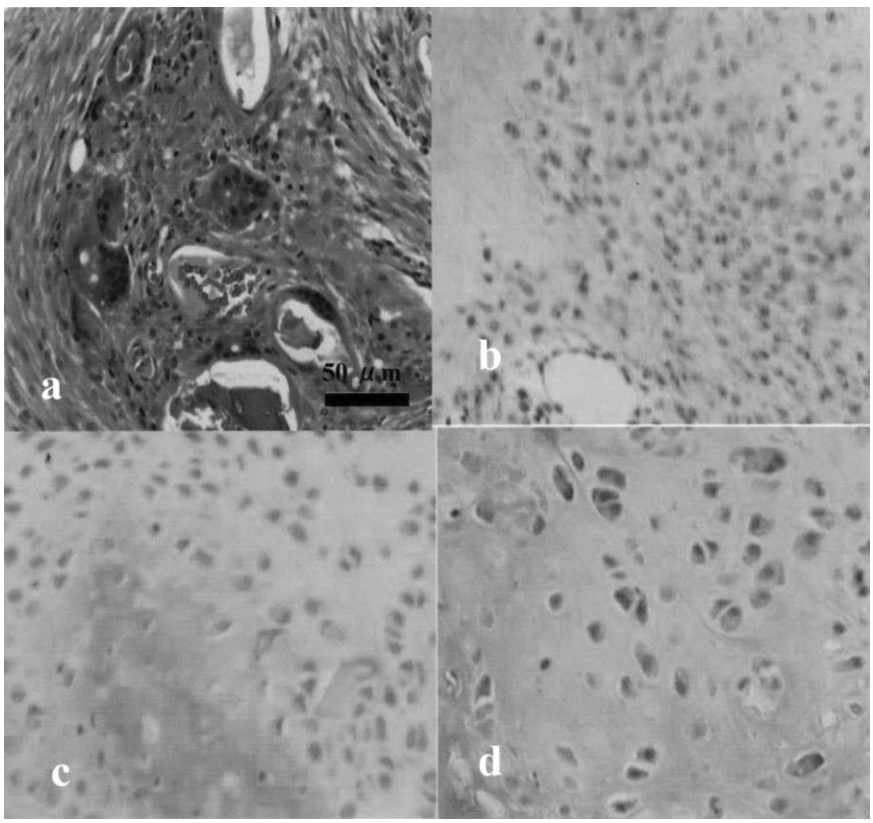

Fig. 4. Histological findings 3 weeks after the operation (higher magnification images). (a) The restored part of the bone trabecula of rabbits in the control group. Permeation was found in inflammation cells and osteoclast (marked with arrows). (b) The restored parts of rabbits in the glucose group. Hyalin and fibrous tissue were found in restored part. (c) The parts restored from the injuries of rabbits in the *N*-acetyl-D-glucosamine group. This finding was different from the control and the Glc group. The parts restored from the injuries of rabbits in the glucosamine group. A massive proliferation of cartilage blast cells is observed. (d) The parts restored from the injuries of rabbits in the glucuronic acid group. This finding was different from the control and the Glc group. The parts restored from the injuries of rabbits in the glucosamine group. A massive proliferation of cartilage blast cells is observed.

a result of this, the amount of GlcNAc administered to the animals ranged from 35 to 476 mg/kg.

In our previous report (Tamai et al., 2002) oral administration of D-glucosamine had no effect on weighing. The present data showed the same results, namely Glc, GlcUA and GlcNAc had also no effects on weighing. Calculated energy of each administered dose (1 g) is only about 4 kcal for glucose. GlcUA and GlcNAc are also thought changed into component of GAG and so they had no effects on weighing.

The surgically created artificial injuries in the stifle joint were shown in Fig. 1(a). Fig. 1(b) and (c) shows the stifle joint at 3 weeks after surgery in the control and glucose group. Two holes (the medial and the distal) in Fig. 1(b) (control) were not healed completely. Two holes (the proximal and the distal) in Fig. 1(c) (glucose) were not healed completely. By contrast Fig. 1(d) and (e) shows the findings of the GlcUA and GlcNAc group, 3 weeks after the operation where are all the three injured parts perfectly healed. From the results of the degrees of healing (Table 2) at the medial holes, 4 out of 6 (67%) of cases showed (+++) in the control group and 3 out of 3 (100%) cases in the Glc, GlcUA and GlcNAc groups. For 80–100% healing the proximal holes, 2 out of 3 cases (67%) in the GlcUA group showed 80–100% healing (+++), but only 2 out of 6 (33%) in the control group, 1 out of 3 (33%) cases in the GlcNAc and 0 out of 3 (0%) cases in the Glc group showed (+++). For 80–100% healing the distal holes, 2 out of 3 cases (67%) in the GlcUA and GlcNAc group showed 80–100% healing (+++), but only 2 out of 6 (33%) in the control group and 0 out of 3 (0%) cases in the Glc group showed (+++). This result was quantified using numerical assignment (Table 3). Although there was no significance seen (Mann–Whitney's U test) in injured parts of the medial and the distal holes, we found a significant healing for the proximal holes ($p < 0.05$) and for the total points ($p < 0.05$) of the GlcUA and the GlcNAc group.

Table 4 shows the comparison between the weight of the biceps of the femur and that of the lateral great muscle. As a result of the significance test, with respect to the weight of the lateral great muscle and the biceps of the femur, no significant difference was found among all groups compared with control group.

The present study proved that oral administration of the glucuronolactone and GlcNAc of 1 g/head (400 mg/kg on the average) facilitate healing of artificially created cartilage injuries in rabbits. Particularly, the restoration of the injuries on the trochlear sulcus was remarkable. It is well known that

glucuronolactone changes into GlcUA in water (Mehltretter, 1953). Therefore, oral administered glucuronolactone changed into GlcUA in the stomach and absorbed from the intestine. In present experiment, the dose of GlcUA and GlcNAc did not cause side effects such as diarrhea, appetite loss and weight loss.

The injuries on medial trochlea caused almost no physical stimulus at the injured part when the knee joint was in motion. On the other hand, since the patella's pressure stimulus impinges on the injuries of the trochlear sulcus constantly, the result seems to have highlighted the healing effect of the GlcUA and GlcNAc group in comparison with the control and Glc group.

3.2. Histological findings

In the control group (Figs. 2(b) and 3), the injured parts showed a lack of normal cartilage and bone trabecula, and migration of macrophage, neutrophils and lymphocytes, and the bone crest surface showed permeation of osteoclasts (Fig. 4(a)). Hyperplasia of capillary vessels and proliferation of fibroblasts cells were found, and proliferation of fibrous connective tissues were found in the injured parts, and new generation of cartilage tissues was never found in its most medial part. In the glucose group (Fig. 2(b)), however, matured cartilage tissues and connective tissues were mixed regenerated in the injured parts. On the other hand, matured cartilage tissues were massively proliferated in the GlcUA and the GlcNAc group (Fig. 2(c) and (d)). They were surrounded by the proliferation of undifferentiated blast cells (fibroblast cartilage cells), while the tissue with cartilage substrates was observed. There was no osteoclast in the bone trabecula (Fig. 4(b)–(d)). Among the normal articular cartilage layer, in the GlcUA and GlcNAc group the structure of cartilage cells looked more like the columnar alignment of the growing zone, and the number of cartilage cells was seen to have increased as a whole when compared with the control group. In addition, the hue of the cartilage substrate had a dark bluish color, showing a possible increase in the amount of matrix content, when compared with that of control group. When compared within the growing zone, the thickness of the cells in the supplementary zone appeared thicker with more cells in the GlcUA and GlcNAc than in the control group.

The difference between both of the groups is apparent in terms of the histological findings. The effects of GlcUA and GlcNAc were summarized as follows. (1) GlcUA and GlcNAc increases the number of cartilage cells even in the normal cartilage. (2) With respect to the injured part, the stimulus on it was much stronger and proliferated cartilage blast cells in a massive manner. (3) The injured bone trabecula in the control group was not healed within 3 weeks and migration of osteoclasts was observed. However, in the GlcUA and GlcNAc group perfect restoration has completed and no osteoclast was observed.

3.3. Image analysis

The results of the image analysis of the special staining samples were shown in Tables 5–7. After carrying out image analysis of various restored parts (Table 5), the non-injured articular cartilages (Table 6) and growing zone (Table 7) with the Alcian blue stains (Fig. 2(c) and (e)), the GlcUA and GlcNAc group showed highly significant values in restored parts and the articular cartilages. However, there was no

Table 5
Effect of glucose, glucuronic acid and *N*-acetyl-D-glucosamine on matrix formation at the injured areas by the image analysis of special staining specimens

Group	Alcian blue staining	Safranin O staining
Control		
1	149.94	129.72
2	149.15	129.85
3	149.85	130.49
4	153.08	131.45
5	152.69	130.90
6	147.58	131.22
Mean	150.38	130.61
Glucose		
1	156.73	163.26
2	153.05	165.12
3	153.84	161.15
4	151.87	163.81
5	153.01	165.93
6	152.83	162.95
Mean	153.56	163.70
p		<0.05
Glucuronic acid		
1	157.82	168.29
2	160.34	171.45
3	158.11	169.62
4	159.26	169.78
5	160.88	170.76
6	158.76	168.66
Mean	159.20	169.64
p	<0.05	<0.05
N-acetyl-D-glucosamine		
1	154.06	166.58
2	153.74	165.82
3	153.62	164.45
4	154.29	165.47
5	153.51	166.15
6	152.43	164.68
Mean	153.61	165.53
p	<0.05	<0.05

Pixels: the 200 times magnified images of restored parts with Safranin O stains and Alcian blue stains were taken into the computer Photograb ab-300 and the images digitized by using Adobe Photoshop. Total pixels 120,000 pixels (random sampling of 20,000 pixels at 6 locations) were calculated through the image processing technique.

significance in the growing zone. Carrying out same image analysis of various parts on the Safranin O stains (Fig. 2(d) and (f) and Tables 5–7), the GlcUA and GlcNAc group showed higher values in the restored part, in the articular cartilage. However, there was no significance in the growing zone.

The difference between both of the groups is apparent in terms of the histological findings by special staining. From the special staining sample of matrix, there was an overwhelming increase in the content by the amount of PG and that of GAG in the GlcUA and GlcNAc group.

In human, the efficiency of GlcNAc against arthritis was reported when its oral administration of 1.0–1.5 g/head (John et al., 1996). And there is no report on the efficacy volume of GlcUA for osteoarthritis treatment. Regarding optimal dose of GlcUA and GlcNAc, further study will be necessary.

Shikman, Kuhn, Alaaeddine, and Lotz (2001) proved in vitro that GlcNAc inhibited IL-1β and TNFα production and induced NO production in normal human articular chondrocytes. In addition, it was shown that GlcNAc also

Table 6
Effects of glucose, glucuronic acid and *N*-acetyl-D-glucosamine on matrix formation at the normal cartilage of trochlear sulcus of femur by the image analysis of special staining specimens

Group	Alcian blue staining	Safranin O staining
Control		
1	148.67	152.30
2	149.25	153.93
3	150.24	154.28
4	154.26	151.98
5	154.20	154.53
6	152.35	149.34
Mean	151.50	152.73
Glucose		
1	154.91	166.06
2	148.36	164.21
3	150.95	165.93
4	150.99	169.15
5	150.14	162.65
6	154.07	162.61
Mean	151.57	165.09
p		<0.05
Glucuronic acid		
1	170.49	201.56
2	169.14	204.64
3	169.19	201.19
4	173.54	198.08
5	173.04	196.88
6	172.21	199.15
Mean	171.27	200.25
p	<0.05	<0.05
N-acetyl-D-glucosamine		
1	168.88	173.86
2	166.12	174.42
3	168.05	173.67
4	169.03	174.19
5	169.24	174.56
6	169.81	173.77
Mean	168.52	174.08
p	<0.05	<0.05

Pixels: the 200 times magnified images of restored parts with Safranin O stains and Alcian blue stains were taken into the computer Photograb ab-300 and the images digitized by using Adobe Photoshop. Total pixels 120,000 pixels (random sampling of 20,000 pixels at 6 locations) were calculated through the image processing technique.

Table 7
Effects of glucose, glucuronic acid and *N*-acetyl-D-glucosamine on matrix formation at the growing zone by the image analysis of special staining specimens

Group	Alcian blue staining	Safranin O staining
Control		
1	167.59	184.90
2	169.84	187.62
3	171.74	193.85
4	175.72	186.59
5	175.25	183.02
6	173.96	180.39
Mean	172.35	186.06
Glucose		
1	171.13	188.79
2	170.96	187.76
3	173.01	188.24
4	166.11	185.12
5	171.61	186.19
6	172.65	186.82
Mean	170.91	187.15
p	0.34	0.28
Glucuronic acid		
1	175.81	195.96
2	176.85	194.91
3	172.43	194.86
4	172.99	194.27
5	175.47	194.63
6	175.25	195.11
Mean	174.80	194.96
p	< 0.05	<0.05
N-acetyl-D-glucose		
1	174.18	195.14
2	172.94	195.86
3	174.19	194.66
4	173.88	197.52
5	173.92	196.24
6	175.26	196.46
Mean	174.06	195.98
p	< 0.05	<0.05

Pixels: the 200 times magnified images of restored parts with Safranin O stains and Alcian blue stains were taken into the computer Photograb ab-300 and the images digitized by using Adobe Photoshop. Total pixels 120,000 pixels (random sampling of 20,000 pixels at 6 locations) were calculated through the image processing technique.

suppressed the production of IL-1β and induced cyclooxygenase-2 and IL-6 from the chondrocytes. The effect of the sugars on NO production is specific, since several other sugars, including glucose, GlcUA and GlcNAc polymers, do not express this activity. With regard to the effect of neutrophil, GlcNAc did not affect the neutrophil functions as superoxide generation, phagocytosis, granule enzyme release and chemotaxis (Hua, Sakamoto, & Nagaoka, 2002). GlcNAc showed a mild inhibitory effect on elastase enzyme release, however, it too acted in a dose fashion. The range of inhibition was 8.18–17.17% (Kamel, Hanafi, & Bassiouni, 1991). GlcNAc is already known to have the action of anti-inflammation without potential dependence on cyclooxygenase, which is different from regular anti-inflammatory agents, as well as the action of free radical's scavenger (Sato et al., 1988). Karzel and Domenjoz (1971) speculated that administration of externally generated GlcNAc directly transform it into *N*-acetylglucosamine-6-phosphoric acid and then into hyaluronic acid or keratan sulfate (Karzel & Domenjoz, 1971). Although the previous was only speculation, the present experiment was able to verify these ideas in the in vivo experiment. With respect to the facilitation of restoration of the bone crest and enhancing healing of the bone crest by GlcNAc was proved in the experiment. The author had proved same effect by D-glucosamine (Tamai et al., 2002). Glucuronolactone is a common component of GAG except for keratan sulfate. Moreover, it is clear that it is used in the synthesis of matrix. From the present results, glucuronolactone will be a most effective supplement for prevention of degenerative joint disease. Further investigation will be necessary for improving exogenous sugars such as glucuronolactone, etc. to be used directly for synthesis of GAG.

With regard to the regeneration of cartilage, Mitchell and Shepard (1976) reported that the tissues observed 1 year after the injury showed the characteristics of fibrous cartilage-like tissues rather than cartilage-like tissues. In addition, Furukawa, David, Koide, Melvin, and Glimcher (1980) reported that there was a large amount of variance in the hexosamine content of the restored cartilage (galactosamine/glucosamine). According to the report, although the restored tissues resemble hyaline cartilage due eventually to the reduction in PG, complete healing cannot be attained because of the mixture of fibrous cartilage and hyaline cartilage. Since the present experiment did not investigate the collagen content in the restored tissue, it is impossible to decide whether the restored tissue will be mature hyaline cartilage or not. In the future study, a longer period of observation and an analysis of regenerated cartilage are necessary.

As a model of restoration of the knee joint, the present experiment made it possible to acquire useful information without difficulty and estimate an appropriate evaluation of administered substances. From the result of the present experiment, we can conclude that GlcUA and GlcNAc facilitate healing of mechanical injuries on cartilage and that GlcUA and GlcNAc increase the amount of GAG and that of PG. Since GlcUA and GlcNAc increased GAG and PG

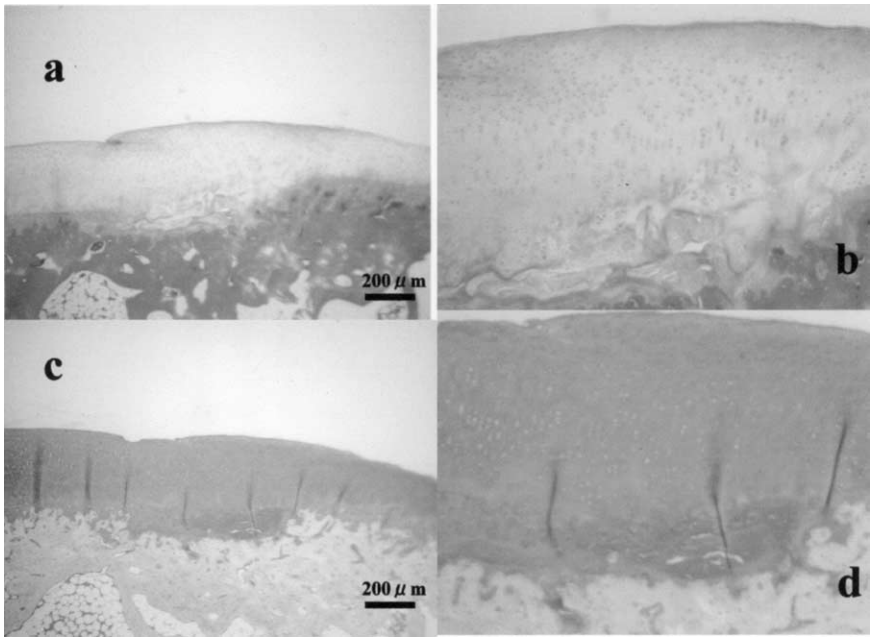

Fig. 5. Follow-up histological findings over 6 months after experimental cartilage injury in a rabbit treated with D-glucosamine hydrochloride (GlcN). All pictures showed regeneration of hyaline cartilage and subchondral trabecular bone at the injured site. Proteoglycan (Safranin-O positive) is also observed in the regenerated cartilage in Photos (c) and (d). Photo (a): Histological findings at the injured site (marked by a square) of the articular cartilage after administration of GlcN for 6 months (hematoxylin and eosin staining, lower magnification). Photo (b): Higher magnification of Photo (a) (the area in the square). Photo (c): The same specimen after staining by Safranin-O. Photo (d): Higher magnification of Photo (c) (the area in the square).

even in the normal tissues, we speculate that glucuronolactone and GlcNAc may be useful for the prevention of articular cartilage injury due to aging and for players and athletes who excessively use their joints. While GlcNAc has been drawing attention recently as an oral agent that can improve pathology of osteoarthritis (Rubin, Talent, Pertusi, Forman, & Gracy, 2001), the present paper is the first ever report, we know of, which proved its effect experimentally. And we also proved the same enhanced healing effect on cartilage injuries by glucuronolactone in this experiment. Systemically, GlcUA and GlcNAc would be used effectively for synthesis of extracellular matrix polymers.

It is important to investigate the fate of regenerated cartilage after administration of GlcUA and GlcNAc. However, we do not have follow-up data for these reagents yet. We have 6 months trial data for GlcN. Histological findings over 6 months of GlcN administration after experimental cartilage damage are shown in Fig. 5 (unpublished data). The method of cartilage damage and the daily dose were the same as in the present study. At the injured site, regeneration of hyaline cartilage and subchondral trabecular bone were observed. From the results, the regenerated cartilage maintained its function for 6 months. In general, it is well known that injured cartilage is not replaced by hyaline cartilage, but by fibrocartilage. In fibrocartilage, regeneration of PG is not observed and articular function is poor. The follow-up data clearly show that administration of GlcN effectively prevents fibrocartilage formation at the injured site and induces hyaline cartilage with regeneration of chondroblasts. Our speculation is that GlcUA and GlcNAc will also maintain hyaline cartilage-like GlcN and show much benefit for cartilage repair. These agents may be effective for the prevention and treatment of degenerative joint disease. However, more experiments with a longer follow-up period to investigate GlcN, GlcUA and GlcNAc will be necessary.

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