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# In vivo effects of isolated implantation of salmon-derived crosslinked atelocollagen sponge into an osteochondral defect

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**Abstract** We have developed crosslinked salmon-derived atelocollagen (SC) sponge, which has a denaturation temperature of 47°C. Sixty-four knees of 32 mature rabbits were randomly divided into 4 groups after creating an osteochondral defect in the femoral trochlea. Defects in Groups I, II, and III were filled with the crosslinked SC sponge, the crosslinked porcine collagen (PC) sponge, and the non-crosslinked PC sponge, respectively. In Group IV, defects were left untreated as the control. At 12 weeks after implantation, the histological score showed that Group I was significantly greater than Groups III ($P = 0.0196$) and IV ($P = 0.0021$). In addition, gene expression of type-2 collagen, aggrecan, and SOX9 was the greatest in Group I at 12 weeks. The fundamental in vivo properties of the crosslinked SC sponge showed that this is a promising biomaterial, specifically as a scaffold for cartilage tissue engineering.

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## 1 Introduction

Atelocollagen exhibits excellent biocompatibility, low antigenicity, and high biodegradability as a scaffold for tissue engineering when compared with synthetic polymers [1]. These advantages allow for the use of collagen in medical applications. The primary sources of atelocollagen are the skin or bone of domestic animals, including calves and pigs. These sources, however, carry potential risks of disease (zoonosis) transmission, such as bovine spongiform encephalopathy (BSE), Foot-and-mouth disease, and so on, to humans. These risks have been recognized as serious obstacles in the clinical field. Recently, fish-derived atelocollagen has attracted great notice as an alternative to animal collagen, because no diseases that can transmit from fishes to humans have been found. In addition, fish collagen has a potentially large pool as a source [2]. Fish-derived atelocollagen, however, has not yet been used as a biomaterial in medical applications, because of its low denaturation temperature. Namely, fish collagen denatures and melts at the physical temperature of the human body [3]. The thermal stability of fish collagen must be improved before it can be used as a biomaterial. We extracted salmon collagen from the fresh skin of chum salmon (*Oncorhynchus keta*), which is discarded as industrial waste in fishing industries, and we previously reported a method of improving the thermal stability of the salmon atelocollagen (SC) gel [4, 5]. However, the denaturation temperature was about 19°C. In our recent study, however, we have overcome the thermal stability problem by synergistic effects of cross-linking and collagen fibril formation [4]. Fibril formation involves the aggregation and alignment of collagen molecules, and the formation helps increase the collagen's thermal stability. The introduction of cross-linking using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)

during fibril formation further increases the denaturation temperature in the SC gel to 47°C. Thus, the crosslinked SC as a biomaterial must be evaluated by in vitro and in vivo studies. Recently, we have evaluated a crosslinked SC gel in the in vitro condition using human periodontal ligament fibroblasts (HPLFs) and human umbilical vein endothelial cells (HUVECs), and reported that the cells could grow and show highly differentiated activity on the SC gel as well as on the porcine collagen (PC) gel [4, 6, 7]. Few studies, however, have evaluated the fundamental properties of the crosslinked SC as a scaffold in the in vivo condition.

To conduct in vivo studies, we have newly developed crosslinked SC sponge that we can easily implant as a scaffold in vivo. In the present study, we have intended to evaluate the fundamental properties of the crosslinked SC sponge as a scaffold for in vivo cartilage repair, because collagen scaffolds have been widely used for cartilage repair [8–11]. In the previous studies [9–11], we have found that scaffolds have effects that slowly induce cartilage tissue regeneration. For example, Dorotka et al. [11] reported that implantation of the three-dimensional PC matrix without any seeded cells showed moderate effects in cartilage regeneration, although the effects were observed to a lesser degree than the cell-seeded collagen matrix. Therefore, to evaluate fundamental properties of the SC sponge as a scaffold in vivo, we have implanted only the sponge in a rabbit osteochondral defect and compared the effect of the implantation between the SC and PC sponges. Based on the above-described in vitro studies [6, 7], we have hypothesized, first, that the crosslinked SC sponge implanted into an osteochondral defect created in the rabbit knee may not show any detrimental effects in comparison with the PC sponge, and second, that the implantation of the crosslinked SC sponge may enhance cartilage regeneration in vivo at 12 weeks after implantation. The purpose of this study is to test these hypotheses.

## 2 Materials and methods

### 2.1 Preparation of collagen sponge

Salmon atelocollagen was prepared from the fresh skin of chum salmon by acid solubilization and subsequent pepsin digestion, according to a procedure reported previously [12]. Briefly, the lyophilized SC was added to 100 ml of diluted HCl (pH 3) to a concentration of 0.5% (w/v) and stirred overnight at 4°C to create the SC solution. Next, 1 ml of the SC solution was transferred into a multiple 48-well plate for tissue culture (Asahi Techno Glass, Tokyo, Japan) and then frozen at −70°C for 12 h. The frozen plates were placed into a lyophilizer (FDU-830, Tokyo Rikakikai, Tokyo, Japan) for 24 h. The porous SC materials obtained were immersed in 4-M NaCl aqueous solution containing 1% (w/v) water-soluble EDC (Dojindo, Tokyo, Japan) and placed at 4°C for 24 h. The crosslinked SC materials were washed three times with diluted water and then lyophilized again. Thus, we obtained sponge-like SC materials. PC solution (Nitta Gelatin, Osaka, Japan) was purchased, and the crosslinked PC sponge was prepared in the same manner as described above. Thus, we prepared three types of sponge: crosslinked SC sponge, non-treated PC sponge, and crosslinked PC sponge. From these materials, we created cylindrical blocks having a diameter of 4.5 mm and a height of 3 mm (Fig. 1).

### 2.2 Degree of crosslinking

The degree of crosslinking of the crosslinked SC and PC sponges was measured as the decrease in the free amine group contents of collagen molecules. Collagen gel samples ($n = 3$ for each sponge) were frozen at −80°C for 12 h, and then lyophilized for 24 h. In the lyophilized samples, the free amine group content was measured spectrophotometrically after reaction of the free amine groups with 2,4,6-trinitro-benzensulphonic acid (Wako Pure Chemical Industries, Osaka, Japan) according to the previously reported method [5], and was expressed as the decrease ratio in the free amine group contents of the crosslinked sample to the uncrosslinked sample.

### 2.3 Study design and animal experimentation

Animal experiments were carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine under the Rules and Regulation of the Animal Care and Use Committee. A total of 32 mature female Japanese white rabbits, weighing 3.9 ± 0.5 kg, were used in this study. An operation for each animal was performed under intravenous anesthesia (pentobarbital, 25 mg/kg) under sterile conditions. In each animal, the bilateral knee joints were exposed through a medial parapatellar incision, and the patella was dislocated laterally. A full-thickness osteochondral defect having a diameter of 4.3 mm and a depth of 3 mm was created in each femoral trochlea, using a sharp drill. A total of the 64 defects in the 32 rabbits were randomly divided into 4 groups (n = 16) depending on the treatment. All defects in Groups I, II, and III were filled with the crosslinked SC sponge, the crosslinked PC sponge, and the non-crosslinked PC sponge, respectively (Fig. 2). In the remaining group (Group IV), defects were left untreated as the control. The incised joint capsule and the skin wound were closed in layers with 3-0 nylon sutures, and an antiseptic spray dressing was applied.

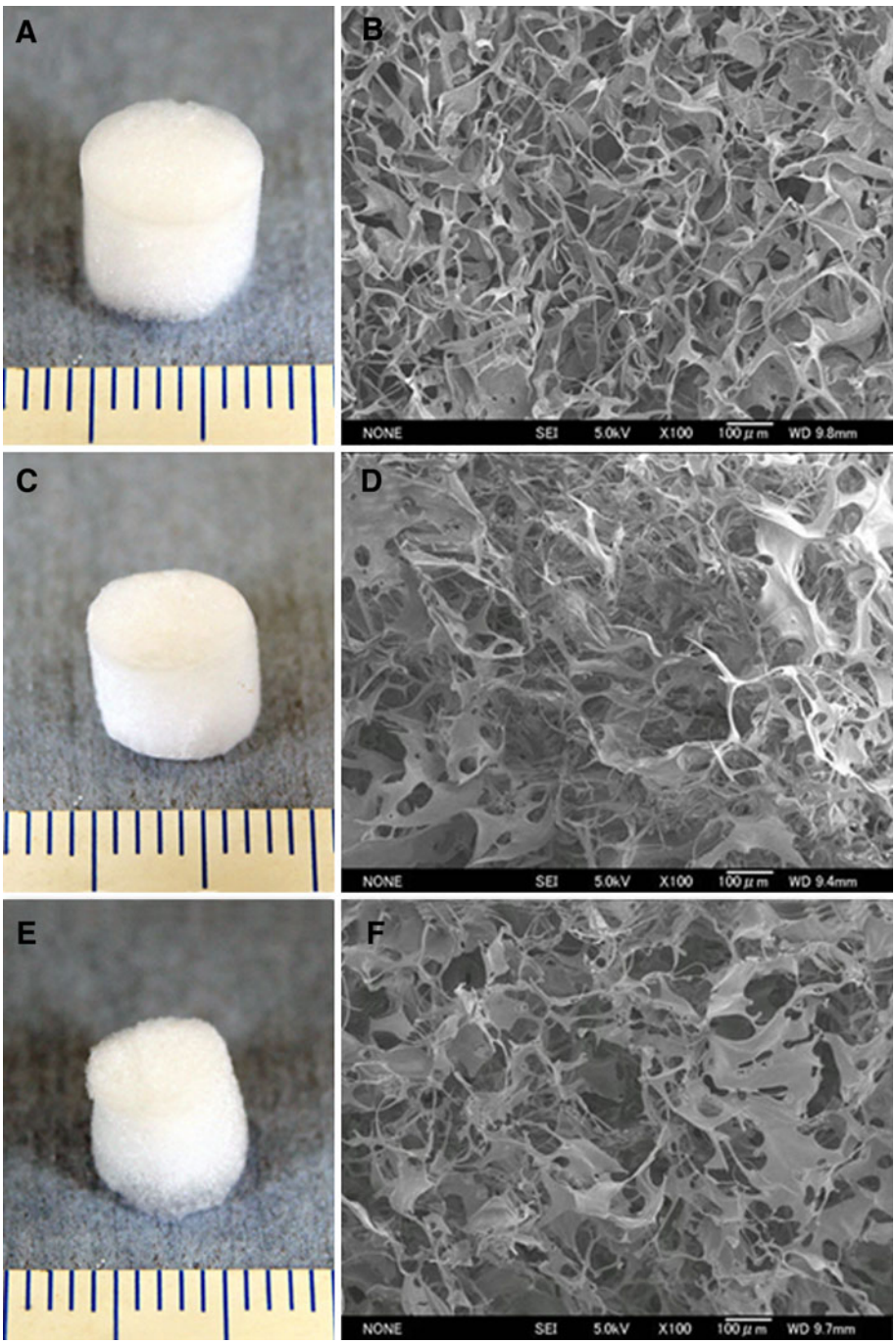


**Fig. 1** Cylindrical collagen sponge blocks and their photographs taken with scanning electron microscope: Crosslinked salmon collagen sponge (**a**, **b**), crosslinked porcine collagen sponge (**c**, **d**), and non-crosslinked porcine collagen sponge (**e**, **f**)

Postoperatively, each animal was allowed unrestricted activity in a cage (310 × 550 × 320 mm) without any joint immobilization. Sixteen rabbits were sacrificed at 4 weeks after surgery, and the remaining 16 rabbits were sacrificed at 12 weeks. The knee joints were immediately removed en bloc with the surrounding tissue. In each group, 6 knees underwent gross, histological, and immunohistochemical evaluations, and the remaining 2 knees were used for real-time polymerase chain reaction (PCR) analyses.

### 2.4 Gross observations and histological evaluations

*At the time of sacrifice*, the tissue regenerated in the osteochondral defect underwent gross observations, and was quantitatively evaluated with the grading scale reported by Wayne et al. [13]. The maximum total score was 16 points.

A distal portion of the resected femur was fixed in a 10% neutral buffered formalin solution for 3 days, decalcified with 50 mM ethylenediaminetetraacetic acid (EDTA) for a

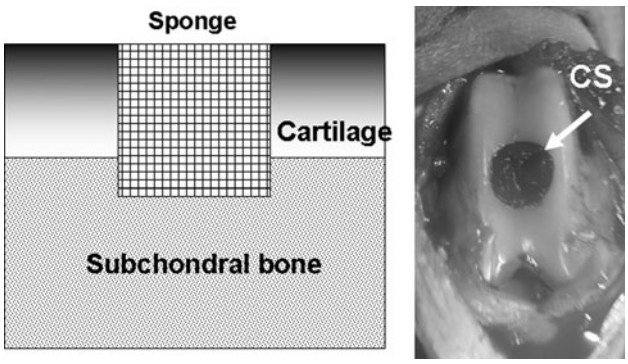


**Fig. 2** A full-thickness osteochondral defect having a diameter of 4.3 mm and a depth of 3 mm was created in the femoral trochlea. The defect was filled with the collagen sponge (CS)

period of 3–4 weeks, and then cast in a paraffin block. The femur was sectioned perpendicular to the longitudinal axis, and stained with Hematoxylin and eosin and Safranin-O. For immuno-histological evaluations, monoclonal antibody (anti-hCL(II), purified IgG, Fuji Chemical Industries Ltd, Toyama, Japan) was used as primary antibodies. Immunostaining was carried out according to the manufacturer's instructions using the Envision immunostaining system (DAKO Japan, Kyoto, Japan). Finally, the sections were counterstained with hematoxylin. Histology was evaluated with a scoring system reported by Wayne et al. [13]. Subsequently, the maximum total score was 19 points.

### 2.5 Real-time PCR analysis

Total RNA was extracted from the tissues regenerated in the defect using the RNeasy mini kit (Qiagen Inc., Valencia, CA) at 4 and 12 weeks, respectively. RNA quality from each sample was assured by the A260/280 absorbance ratio. The RNA (100 ng) was reverse-transcribed into single strand cDNA using PrimeScript® RT reagent Kit (TakaraBio, Ohtsu, Japan). The RT reaction was carried out for 15 min at 37°C and then for 5 s at 85°C. All oligonucleotide primer sets were designed based upon the published mRNA sequence. The expected amplicon lengths ranged from 93 to 189 bp. The sequences of primers used in real time PCR analyses for rabbit regenerative tissues were as follows: type 2 collagen forward GACCATCAATGGCGGCTTC; reverse CACGCTG TTCTTGCAGTGGTAG. Aggrecan forward GCTACGAC GCCATCTGCTAC; reverse GTCTGGACCGTGATGTC CTC. SOX9 forward AACGCCGAGCTCAGCAAGA; reverse TGGTACTTGTAGTCCGGGTGGTC. GAPDH forward CCCTCAATGACCACTTTGTGAA; reverse AGGCCATGTGGACCATGAG. The sequences of primers used in real time PCR analyses for mouse ATDC5 cells were as follows: type 2 collagen forward AGGGCAACAG CAGGTTCACATAC; reverse TGTCCACACCAAATTC CTGTTCA. Aggrecan forward AGTGGATCGGTCTGAA TGACAGG; reverse AGAAGTTGTCAGGCTGGTTT GGA. SOX9 forward GAGGCCACGGAACAGACTCA; reverse CTTCAGATCAACTTTGCCAGCTT. GAPDH forward TGTGTCCGTCGTGGATCTGA; reverse TTGCT GTTGAAGTCGCAGGAG. The real-time PCR was performed in Thermal Cycler Dice® TP800 (TakaraBio, Ohtsu, Japan) by using SYBR® Premix Ex Taq™ (TakaraBio, Ohtsu, Japan). cDNA template (5 ng) was used for real-time PCR in a final volume of 25 μm. cDNA was amplified according to the following conditions: 95°C for 5 s and 60°C for 30 s at 40 amplification cycles. Fluorescence changes were monitored with SYBR Green after every cycle. A dissociation curve analysis was performed (0.5°C/s increase from 60 to 95°C with continuous fluorescence readings) at the end of cycles to ensure that single PCR products were obtained. The amplicon size and reaction specificity were confirmed by 2.5% agarose gel electrophoresis. The results were evaluated using the Thermal Cycler Dice® Real Time System software program (TakaraBio, Ohtsu, Japan). Glyceroaldehyde-3-phosphate dehydrogenase (GAPDH) primers were used to normalize samples.

### 2.6 Statistical analysis

All data were described as the mean and standard deviation values. The one-way analysis of variance (ANOVA) was performed only at 12 weeks with the Fischer's PLSD test for post hoc multiple comparisons, because the 4-week period was too early to evaluate cartilage regeneration. The significance limit was set at $P = 0.05$. For calculation, the StatView 5.0 software (SAS Institute Inc., Cary, NC, USA) was used.

## 3 Results

### 3.1 Degree of crosslinking

The degree of crosslinking (the decrease ratio in the free amine group contents of the crosslinked sample to the uncrosslinked sample) of the crosslinked SC and PC sponges averaged 41.3% (SD 8.2) and 43.4% (SD 2.7), respectively.

### 3.2 Gross observation

In gross observation, we did not observe any inflammatory findings or any pathological changes in each knee joint at each period. At 4 weeks, the defects in each group were filled with white fibrous tissues (Fig. 3). At 12 weeks, the

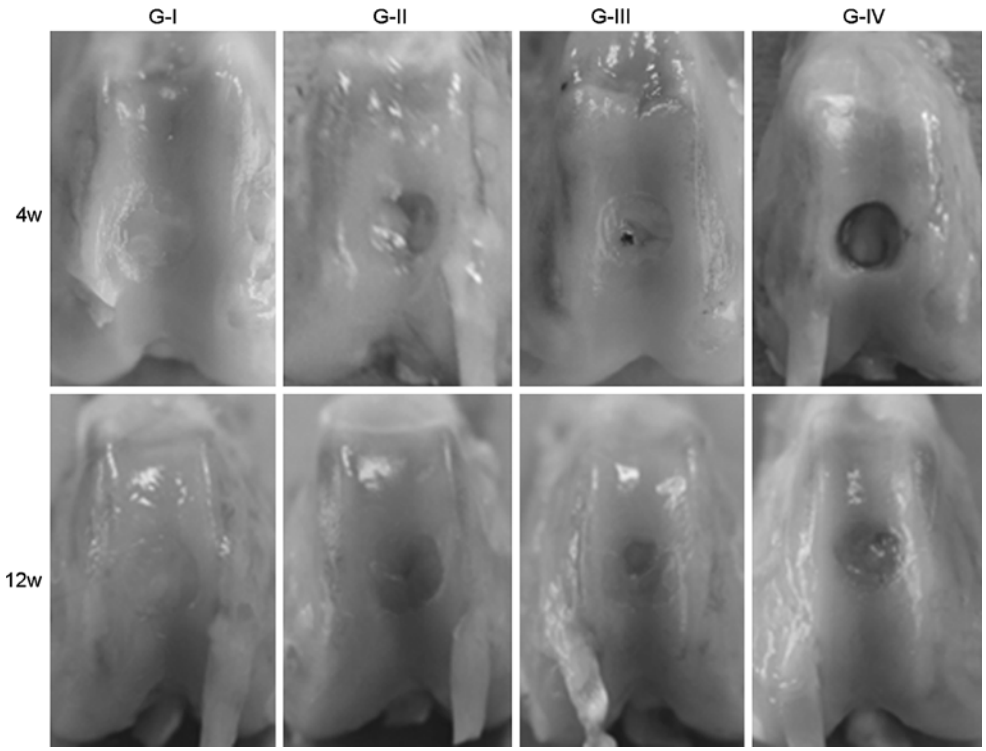


**Fig. 3** In gross observation at 4 weeks, the defects in Groups I, II, and III were filled with white fibrous tissues. At 12 weeks, the defects in Groups I (crosslinked SC) and II (crosslinked PC) were filled with a white opaque tissue, while the defects in Groups III (non-crosslinked PC) and IV (non-treated) showed reddish opaque, patchy, stiff tissues

defects in Groups I and II were filled with a white opaque elastic tissue, while the defects in Groups III and IV showed reddish patchy tissues (Fig. 3).

Concerning the Wayne's score at 12 weeks, the ANOVA showed a significant difference ($P = 0.0378$). The post-hoc test indicated that Group I was significantly greater than Group IV ($P = 0.0051$), while Group II or III was not significantly greater than Group IV (Fig. 4a).

### 3.3 Histological and immunohistochemical observations

At 4 weeks, each defect was filled with fibrous tissues (Fig. 5A–D). We occasionally and irregularly observed a small amount of a proteoglycan-rich tissue in the tissues in Group I. In Group IV, bone formation with a small amount of a proteoglycan-rich tissue was seen.

At 12 weeks, the defects in Groups I and II were filled with a proteoglycan-rich tissue, while the width of this tissue was approximately a half of the normal articular cartilage (Fig. 5E, F). In the proteoglycan-rich tissue, type 2 collagen was abundantly expressed (Fig. 5I, J). The defects in Groups III and IV were filled with the fibrous and bone tissues (Fig. 5G, H), while a small amount of the proteoglycan-rich tissue was occasionally and irregularly seen in these tissues. The type 2 collagen expression was not found in the tissue regenerated in the untreated defect, except for a limited amount in the peripheral portion (Fig. 5K, L). In high magnification histology of Group I, no cleft was observed between the regenerated tissue and the normal cartilage tissue (Fig. 5a). In the regenerated tissue, fairly large round cells rich in cytoplasm were scattered singly or as an isogenous group in a proteoglycan-rich matrix (Fig. 5b). At the superficial layer in this tissue, cells were rather small and sparse, resembling the lamina splendens in the normal articular cartilage (Fig. 5c).

Concerning the histological score at 12 weeks (Fig. 4b), the ANOVA showed a significant difference ($P = 0.0378$). The post-hoc test indicated that Group I was significantly greater than Groups III ($P = 0.0196$) and IV ($P = 0.0021$). Group II was significantly greater than Group IV ($P = 0.0121$). There was no significant difference between Groups III and IV.

### 3.4 Real-time PCR analysis

In the real time PCR analyses, expression of type 2 collagen, aggrecan, and SOX9 mRNAs in Group III was similar to that in Group IV (the non-treated control). Regarding Group I, the relative expression level of type 2 collagen, aggrecan, and SOX9 mRNAs was obviously (2–3 times as much as) higher than that in Group IV (Fig. 6).

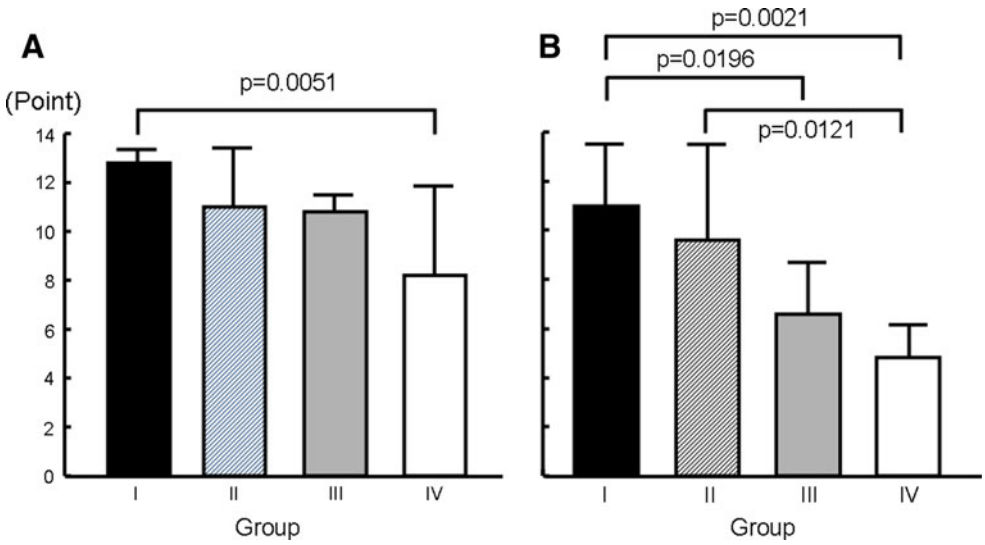


**Fig. 4** Concerning the gross observation score (**a**) at 12 weeks, only Group I was significantly greater than Group IV. Regarding the histological score (**b**) at 12 weeks, Group I was significantly greater than Groups III and IV, while Group II was significantly greater than Group IV. There was no significant difference between Groups III and IV

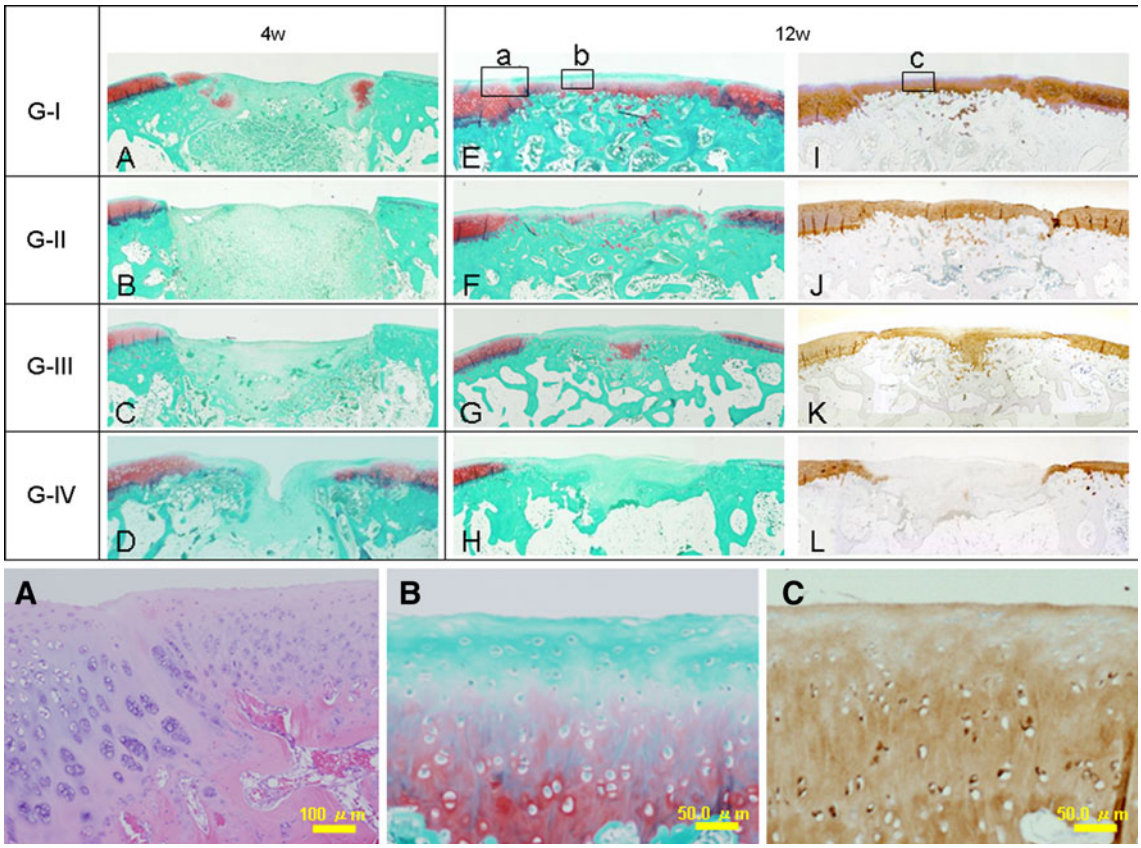


**Fig. 5** At 4 weeks, each defect was filled with fibrous tissues (**A**–**D**). At 12 weeks, the defects in Groups I and II were filled with a proteoglycan-rich tissue (**E**, **F**), in which type 2 collagen was abundantly expressed (**I**, **J**). The defects in Groups III and IV were filled with the fibrous and bone tissues (**G**, **H**, **K**, **L**). **A**–**H**: Safranin-O and fast green staining, original magnification ×2, **I**–**L**: type II collagen staining, original magnification ×2. Three high magnification photographs (**a**, **b**, and **c**) in the bottom row show the tissues surrounded by *rectangular lines*, **a**, **b**, and **c**, in the photographs (**E**) and (**I**). In Group I at 12 weeks, no cleft was observed between the regenerated tissue and the normal cartilage tissue (**a**: A *black bar* shows 100 μm). Fairly large round cells rich in cytoplasm were scattered singly or as an isogenous group in a proteoglycan-rich matrix (**b**: A *black bar* shows 50 μm), while cells were rather small and sparse at the superficial layer, resembling the lamina splendens in the normal articular cartilage. In the proteoglycan-rich tissue as well as the large round cells, type 2 collagen was abundantly expressed (**c**: A *black bar* shows 50 μm)

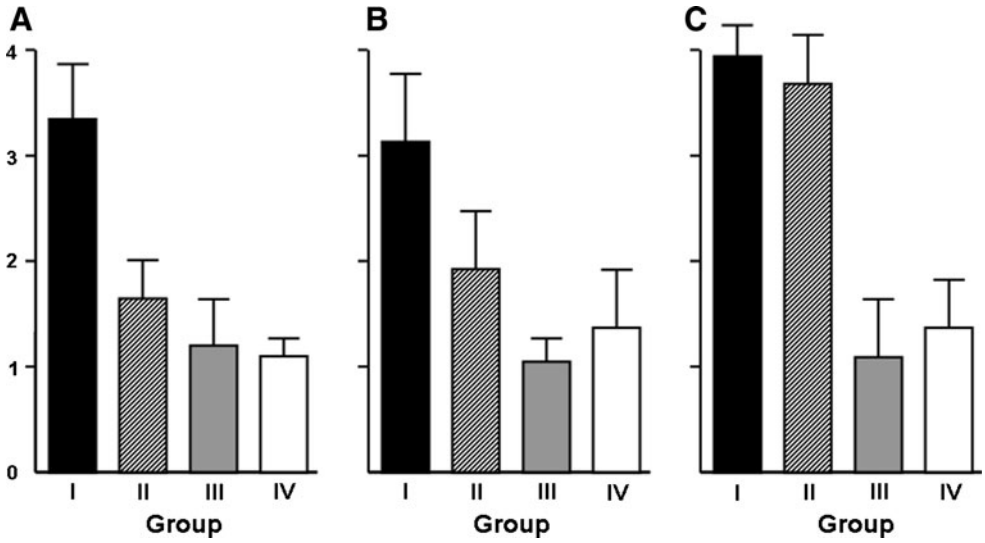


**Fig. 6** In the real-time PCR analysis, mRNA expression of type-2 collagen (**a**), aggrecan (**b**), and SOX9 (**c**) was higher in Group I than in Groups II, III, and IV

Concerning Group II, the SOX9 mRNA expression level was obviously (3 times as much as) higher than that in Group IV (Fig. 6), while the relative expression level of type 2 collagen and aggrecan mRNAs was slightly higher than that in Group IV.

## 4 Discussion

First of all, the present study showed that the crosslinked SC sponge as well as the crosslinked and non-crosslinked PC sponges did not show any detrimental effects in the knee joint, when they were implanted into an osteochondral defect created in the rabbit knee. This result shows that the crosslinked SC sponge can be used as a safe scaffold from the viewpoint of the immunological reaction, because the PC sponge has been established as a useful scaffold material for tissue engineering [1]. It was noted that the crosslinked SC sponge was completely absorbed at 12 weeks, while the crosslinked PC sponge still remained unabsorbed in the defect. This fact indicates that the absorption rate of the crosslinked SC sponge was higher than the crosslinked PC sponge.

Secondly, the present study also demonstrated that implantation of the crosslinked SC as well as the crosslinked PC sponge had a significant effect that induced slow regeneration of the hyaline cartilage in an osteochondral defect at 12 weeks in comparison with the control. This effect was not observed at 4 weeks. In contrast, the commonly available non-crosslinked PC sponge implantation did not have such effects. Additionally, not only the Wayne's scores but also gene expression of type-2 collagen, aggrecan, and SOX9 indicated a tendency that the induction effect of hyaline cartilage regeneration at 12 weeks was the most obvious in the crosslinked SC sponge. This tendency is supported by our previous in vitro study: Namely, HPLFs showed better growth and higher alkaline phosphatase activity in culture on the SC gel than in culture on the PC gel [6]. Thus, we believe that these are important information for the SC sponge as a potential biomaterial.

We should note the differences in biological functions between the SC and the mammalian-derived collagens, although they have not been completely clarified. First, the hydroxyproline content of SC is about half of that of PC [6]. The low content of hydroxyl group in SC leads to a higher hydrophobicity of SC than that of PC. Secondly, manner of integrin-mediated cell attachment to a collagen peptide is different between the SC and other collagens, For example, Nagai et al. [7] showed that HUVEC attachment to the SC gel depends on the alpha v beta 3 integrins as well as the alpha 2 beta 1 integrin, while the HUVEC attachment to the bovine collagen gel depends on the alpha 2 beta 1 integrin. Integrins not only mediate cell adhesion to extracellular matrix but also activate intracellular signaling pathways [14].

In the present study, both the crosslinked SC and PC sponges showed significant effects on the cartilage regeneration in comparison with the control group, while the non-crosslinked PC sponge did not show any significant effects. This fact suggests that an increase of the degree of crosslinking is one cause of the effect. We speculated on the mechanisms. First, crosslinking reduces free-amino residues of collagen molecules. In one of our previous reports, the degree of reduction of the free-amino residues was about 20% [4]. There are reports that the crosslinking of collagen molecules enhanced the DNA synthesis of epidermal cells [14] and the proliferation of endothelial cells [15]. Therefore, it is possible that the reduction of free-amino residues or the change of chemical properties in

the collagen molecules might affect the cartilage regeneration in the present study. Secondly, crosslinking changes the mechanical properties of the collagen sponges. The mechanical properties of the scaffold are known to affect the cellular functions and morphologies [16]. Therefore, it can be speculated that the difference in the mechanical properties between the crosslinked and non-crosslinked collagen sponges might affect cartilage regeneration in the present study. However, how the above-described differences in the collagen sponges affect the cartilage regeneration is unclear and still under investigation.

There are some limitations in this study. The first limitation is that we did not perform long-term observation of the regenerated cartilage. A long-term evaluation study is needed to be performed immediately after this study. The second limitation is that we could not clarify the mechanism of the in vivo cartilage regeneration induction by the crosslinked collagen sponges. Thirdly, we did not perform statistical analysis concerning the gene expression of type-2 collagen, aggrecan, and SOX9. Fourthly, we did not perform a biochemical analysis.

As to clinical relevance, this study showed that the crosslinked SC sponge is a promising scaffold for cartilage tissue engineering. In addition, the SC sponge has great advantages including no risk of zoonosis transmission, a large source for commercial production, and early absorption. However, the width of this tissue was approximately a half of the normal articular cartilage. Therefore, the crosslinked SC sponge implantation itself cannot be a therapeutic option, if it is simply implanted without any modification. In order to obtain sufficient amount of regenerated cartilage, for example, we should consider a modification that some signaling molecules that enhance cartilage regeneration are added in the crosslinked SC sponge. This is a theme of our next research study.

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