【Title】

Repair of large osteochondral defects with allogeneic cartilaginous aggregates formed from bone marrow-derived cells using RWV bioreactor

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【Abstract】

Objective: To examine the technique of regenerating cartilage tissue from bone marrow-derived cells by three-dimensional (3-D) culture using the rotating wall vessel (RWV) bioreactor.

Methods: 3-D and cylindrical aggregates of allogeneic cartilage with dimensions of 10mm x 5mm (height x diameter) formed by the RWV bioreactor were transplanted into osteochondral defects of Japanese white rabbits (Group-T, n=15). For the control, some osteochondral defects were left empty (Group-C, n=18). At 4, 8, and 12 weeks post-implantation, the reparative tissues were evaluated macroscopically, histologically, and biochemically.

Results: In Group-T at as early as 4 weeks, histological observation, especially via safranin-O staining, suggested that the reparative tissues resembled hyaline cartilage. And we observed no fibrous tissues between reparative tissue and adjacent normal tissues. In the deeper portion of the bony compartment, the osseous tissues were well remodeled. At 4 and 8 weeks post-implantation, the mean histological score of Group-T was significantly better than that of Group-C ($P < 0.05$). The glycosaminoglycans (GAG) / DNA ratio in both groups increased gradually from 4 to 8 weeks and then decreased from 8 to 12 weeks.

Conclusions: We here in report the first successful regeneration of cartilage in osteochondral defects in vivo using allogeneic cartilaginous aggregates derived from bone marrow-derived cells by 3-D culture using the RWV bioreactor.

Key Words: Cartilage, RWV bioreactor, Mesenchymal stem cells, 3-D culture, Tissue engineering

【**Introduction**】 Artificial cartilage fabricated in vitro using techniques of tissue engineering has been employed to repair and regenerate damaged cartilage[1,2,3]. There have been numerous reports in this field on experiments with animals or clinical cases[4,5,6]. However, no treatment has been reported to regenerate either a complete or a long-lasting hyaline cartilage. Our research group has recently examined the application of a rotating wall vessel (RWV) bioreactor that simulates a micro-gravity environment with a low shear stress for regenerating cartilage tissue and successfully established a three-dimensional (3-D) cell culture technique for the formation of large and homogenous cartilaginous aggregates from bone marrow-derived cells without using a scaffold[7]. This bioreactor generates stress through the horizontal rotation of a cylindrical vessel equipped with a gas exchange membrane. The bioreactor compensates for the effect of gravity, resulting in homogenous cell growth and differentiation without sinking, and the cells aggregate and form 3-D tissue[8]. Figure.1, A shows a cartilaginous aggregate formed in the RWV bioreactor according to the method described in our previous paper[7]. Fig.1B shows the result of safranin-O staining of a section of the formed cartilaginous aggregate, which shows that the aggregate is large and homogenous and simulates hyaline cartilage with abundant extracellular matrix. The purpose of this study is to evaluate the usefulness of transplanting cartilaginous aggregates formed from rabbit bone marrow-derived cells using RWV bioreactor.

【Materials and Methods】

Cell culture and formation of cartilage aggregates: All experiments were performed in accordance with the guidelines of the Japanese Government for the care and use of laboratory animals. Bone marrow-derived cells were collected from the femurs and tibias of six female 12-day-old Japanese white rabbits. The femurs and tibias were removed aseptically, cleaned of soft tissues, and washed three times in PBS with antibiotics. Then both ends were removed from the epiphyses, and the marrow was flushed out from the modularly cavity using 20ml of standard medium. Next, the cell suspension was distributed through thirty T-75 culture flasks (BD) with 15 ml of a standard medium and then cultured in a humidified atmosphere containing 95% air and 5% CO_2. The standard medium consisted of Dulbecco's Modified Eagle's Medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS; Sigma) and antibiotics (antibiotic-antimicotic: Gibco BRL). Approximately 3 weeks after the seeding, when the adherent cells grew become over-confluent, at which time they were subcultured by trypsinization, and then resuspended in a rotating medium. The rotating medium consisted of DMEM containing 10%FBS, 50 μ g/ml of ascorbic acid (WAKO), 40 μ g/ml of L-proline, ITS culture supplement (BD), 10^{-7}M dexamethasone (Sigma), 10ng/ml of TGF-β_3 (Sigma), and antibiotics (antibiotic-antimicotic:Gibco BRL). Then, 10ml of the cell suspension (1.5-3.0 x 10^7 cells) was seeded into the RWV bioreactor (RCCS^{TM}-4D system

with 10 ml disposable vessels, Synthecon Incorporated, TX) in a CO_2 incubator, and the rotatory culture was performed for 1 week. The speed of rotation was adjusted manually in order to balance the aggregation of cells in the vessel without allowing the aggregate to sink.

Animal model: Thirty-six female 10-week-old Japanese white rabbits (Tokyo Experimental Animals, Tokyo, Japan), weighing 2.3 to 2.5kg, were used in this study. The experiments were approved by the University Committee for Animal Experimentation. All operations were performed on the left knee joint of the rabbits under a general anesthesia induced by intravenous injections of pentobarbital sodium (0.6ml/kg bodyweight i.v.) and under sterile conditions. The left knee was opened via a medial parapatellar approach and the patella was dislocated laterally to expose the patellar groove of the femur. A hand drill, with depth-controlled bits , was used to create cylindrical defects 5×5 mm in area and 4 mm in depth which did not spontaneously regenerate[9] along the midline of the patella groove. For control, the defects were left empty (Group-C: control group, n=18), whereas 3-D cartilage aggregates with dimensions of 10 mm x 5 mm (height × diameter) were cut into two pieces into the defects without any flap covering (Group-T: transplanted group, n=18). The patella was relocated and the wound was closed in layers using 4-0 nylon sutures. The animals were then caged and allowed to move freely without any splinting after recovery,

before finally being sacrificed at 4 (n=6), 8 (n=6) and 12 (n=6) weeks after the operation. However, two rabbits that died during the anesthetic interval and one infected in the operated knee joint were excluded from this study. Thus, the total number of rabbits used for this study was 33.

Macroscopic assessment: The animals were sacrificed by injecting them with a lethal dose of pentobarbital sodium. At necropsy, the defects were examined macroscopically, and photographed. The surface of the grafts was inspected in terms of color, integrity, contour, and smoothness. The quality of repair and the presence of any gross damage to the articular surface were then recorded.

Histology and Immunohistochemistry: At sacrifice, the upper half of the reparative tissue was cut along the coronal plane from the distal femur and fixed by microwave in 4% paraformaldehyde and 1 % glutaraldehyde in PBS for four days. After decalcification in 0.5M EDTA and 0.1M Tris and NaOH for four weeks, the samples were embedded in paraffin, and sectioned (5 μ m) with a microtome. Using the sections obtained at 4, 8, and 12 weeks in each group, we assessed the central one-third of the reparative tissue. The histological sections, stained with safranin-O and fast green to demonstrate the presence of glycosaminoglycans (GAG) and fibrous tissue, respectively, were observed under an optical microscope (Olympus IX-70) equipped with a CCD camera (CoolSnap cf), and graded using

the O'Driscoll scoring system[10], by two blinded observers. Regarding immunohistochemical staining, the sections were deparaffinized with xylene and then rehydrated with decreasing concentrations of ethanol in solution. They were incubated with 3% H_2O_2 for 10 minutes and proteinase K (Dako, Glostrup, Denmark) for 10 minutes to block any endogenous peroxidase activity. They were then incubated with monoclonal mouse antibodies for type-Ⅰ collagen (clone; M35 Developmental Studies Hybridoma Bank)and type-Ⅱ collagen (clone; 4C11 ONCOGENE) at an optimal dilution in 0.05M tris buffered saline (TBS) for one hour. The sections were rinsed with 0.05M TBS three times for five minutes each and incubated with Envision$^+$ (Dako) for thirty minutes. They were rinsed three times with TBS and then incubated with DAB solution (Dako) for a few minutes.

Biochemical analysis: To examine PG content, levels of GAG in the reparative tissue were measured. The lower half of the reparative tissue was dissected off the central portions of the subdivided femora using a scalpel, and then frozen. The frozen samples were carefully crushed and digested with 1 ml of proteinase K solution overnight at 60℃. The amount of sulfated GAG was measured with the Blyscan Sulfated Glycosaminoglycan Assay Kit (Biocolor, Ltd) according to the manufacturer's recommendations. DNA quantification was carried out with a fluorometric assay using the Hoechst dye H33258 (Sigma B-2883)

Statistical analysis: For the data on histological scores and the GAG/DNA ratio of the

tissue, means and standard deviations were calculated for each group. The two-tailed unpaired Student's t-test with Welch's correction was performed to analyze any significant difference between Group-T and Group-C regarding the histological score and the GAG/DNA ratio. P values of less than 0.05 were considered to be statistically significant.

【Results】

Characterization of cartilage aggregates: Figure.1, A shows an image of a 3-D aggregate formed in the RWV bioreactor after 1 week of culture. The aggregates were not round but slender, though they could be handled with surgical forceps. In size they averaged approximately 10mm×5mm (height×diameter). They resembled well-differentiated hyaline cartilage in cellular morphology and were stained strongly by safranin-O as shown in Figure.1, B. This kind of aggregate was formed repeatedly in the same experiment[7].

Macroscopic findings: All the rabbits could run normally and had a normal range of motion in the operated knee at all experimental times. After the animals were sacrificed, the defects of the patella groove were examined macroscopically. In Group-T and Group-C, no signs of immunological rejection or synovial proliferation were observed in the knees at any time after the operation (at 4, 8 or 12 weeks), although osteophytes were present near the medial side of the femoral condyle at the junction of the patellar and tibial articular area, with 5 cases in Group-C and 3 cases in Group-G. In Group-C, at 4 weeks after the operation, an

ulceration of cartilage was observed in only one case at the medial upper side of the patellar groove. At 4 and 8 weeks, the regenerated area in Group-C contained red, irregular tissue with a depression and the margins of the defects were clearly distinguishable from the surrounding normal cartilage (Fig.2, A B). At 12 weeks, the defects were filled with a white tissue, but the regenerated area still had an irregular appearance with a depression and the margins of the defects were also clearly recognizable (Fig.2, C). By contrast, in Group-T, the regenerated area showed a normal contour as early as 4 weeks after the operation. The defects were covered with a white, glossy, smooth membrane, which resembled articular cartilage at 4 weeks after the operation and was maintained up until weeks 8 and 12 (Fig.2, D E F).

Results of safranin-O fast green staining: In Group-T at as early as 4 weeks, the defects were filled with reparative tissue that resembled hyaline cartilage (Fig.3, A). The cells were round and arranged vertically as columns consisting of a few cells (Fig.3, D). At 8 and 12 weeks, the cells remained their morphology, and the extracellular matrices were strongly stained by safranin-O, suggesting the active production of GAG (Fig.3, B C). The reparative tissue had a smooth surface and there were no fibrous tissues between the reparative tissue and adjacent normal cartilage (Fig.3, E). At 8 weeks, in addition to the presence of the hyaline-like cartilage, enchondral bone was observed in the deeper portion of the reparative

tissue. At 12 weeks, although the surface of the reparative tissue seemed to be slightly irregular, most cells were round. Safranin-O staining was evident throughout the reparative tissue, but in some cases its intensity was slightly reduced in comparison to that of the adjacent normal cartilage. However, the reparative tissue retained its thickness (Fig.3, C). In the deeper portion of the bony compartment, the osseous tissue was well remodeled (Fig.3, F). In contrast, in Group-C at 4 weeks after the operation, the defects were filled with fibrous tissue with an irregular surface, and some fissures were noted in it but were partially stained by safranin-O (Fig.3, G). The junction between the tissue and the adjacent normal cartilage was loose and recognizable. At 8 weeks, the defects were filled with reparative tissue similar to that seen at 4 weeks after the operation, (Fig.3, H). At 12 weeks, the reparative tissue showed a loss in intensity of safranin-O staining and a reduction in thickness (Fig.3, I). In general, the untreated defects filled with disorganized fibrocartilage did not recover to develop an articular surface continuous with the surrounding hyaline cartilage.

I**mmunohistochemical staining for type-Ⅰand -Ⅱcollagen:** The regenerated cartilage in Group-T had greater staining of type-Ⅱcollagen on the matrix as well as in the surrounding normal cartilage at all time points after the operation (Fig.3, J). The staining of type-Ⅰ collagen was less intense in the regenerated cartilage zone , but it was more intense in the calcified zone (Fig.3, K).

Histological scoring of the reparative tissue: For a quantitative histological analysis of the reparative tissue, the sections were examined independently by two observers, and scored according to the grading scale established by O'Driscoll et al.[10] from 0 (worst) to 24 (best; normal). The mean scores of the histological assessments are shown in Figure.4. The mean score of both groups increased gradually from 4 to 8 weeks and then slightly decreased from 8 to 12 weeks. At 4 and 8 weeks after the operation, the mean score of Group-T was significantly better than that of Group-C ($P < 0.05$). At 12 weeks, the mean score of Group-T was not significantly different from that of Group-C.

Biochemical findings: The time course of the change in the GAG / DNA ratio of the reparative tissues is shown in Figure.5. In both groups, the ratio was increased gradually from 4 to 8 weeks and then decreased slightly from 8 to 12 weeks. However, there was no significant change with time or difference between the two groups.

【Discussion】 In the present study, large osteochondral defects in the knee joints of rabbits were successfully repaired with hyaline-like cartilage after the transplantation of allogeneic cartilaginous aggregates formed from bone marrow-derived cells using the RWV bioreactor. As early as 4 weeks after the operation, the defects were filled with reparative tissue that resembled hyaline cartilage. The reparative tissue had a smooth surface and there were no fibrous tissues between the reparative tissue and adjacent normal cartilage. At 8 weeks,

enchondral bone had formed in the deeper portion of the reparative tissue. At 12 weeks, in some cases the intensity of staining with safranin-O was slightly reduced in comparison to that in the adjacent normal cartilage, but the reparative tissue retained its thickness. This is the first report of the rapid regeneration of critical osteochondral defects with allogeneic cartilaginous aggregates formed from bone marrow-derived cells without any scaffold using the RWV bioreactor. There are two important points in this study. First, the cells are derived from bone marrow and mesenchymal stem cells (MSCs). Therefore it is possible for the cells to proliferate in a monolayer culture and then to differentiate into cartilage and bone[11,12]. An additional advantage to using bone marrow-derived cells in a clinical context is that they can be collected through aspiration of both sides of the iliac crest under partial anesthesia, a procedure that is very easy to perform and minimally invasive. Previous study[20] found that synovium was a better cell source than bone marrow-derived cells with regard to the regeneration of cartilage by comparing chondrogenic potential using a pellet culture system i*n vitro*. However, the cartilaginous aggregates formed by the RWV bioreactor are approximately five-fold in diameter larger than those formed in a pellet culture, which is an advantage for clinical application. Moreover, collecting synovium from joints is invasive, and the amount from each joint differs depending on the disease such as osteoarthritis, rheumatoid arthritis, or trauma. Consequently, bone marrow-derived cells are a more

suitable source than the synovium for obtaining MSCs and the RWV bioreactor is a more suitable cell culture system than a pellet culture with regard to the regeneration of cartilage in a clinical context. Second, we used the RWV bioreactor as the 3-D culture system which stimulates chondrogenesis in a simulated micro-gravity environment[7]. In previous reports[2,13], engineered cartilage from MSCs required a scaffold for the cells to remain in the defect and to act as a support for inducing the formation of hyaline cartilage. However, using our technique, it is possible to form large and homogenous cartilaginous aggregates without any scaffold which can thus be transplanted into large osteochondral defects, which do not spontaneously regenerate in the rabbit[9]. These aggregates have already produced an abundance of extracellular matrix and type-Ⅱ collagen which was used to identify the chondrogenic phenotype *in vitro*[7]. It suggests that the aggregates formed in the RWV bioreactor have the character of hyaline cartilage. Rich extracellular matrix embedded chondrocytes to maintain their phenotype protecting them from dedifferentiation. In addition, we had a specific reason to choose aggregates at as early as 1 week after culture in the RWV bioreactor for transplantation. According to our in vitro study[7], the cells of cartilaginous aggregates after 1 week of culture are not mature, but consist of undifferentiated or chondrogenic precursor cells, which might exhibit plasticity to differentiate into another phenotype such as osteoblasts etc. The aggregates of such kinds of cells were influenced

by various biological factors from the host bone marrow side[14,15] and interacted with adjacent cartilage[16,17]. A suitable distribution of mechanical stress and synovial factors[18,19] also influence the lineage of these cells. These characteristics may thus make it possible to rapidly and suitably regenerate cartilage-bone structure *in vivo*. The limitations of this preliminary study are that we used 10-week-old rabbit model and only observed short-term results. And it remains unclear, at this preliminary stage, what precisely occurred at the site of repair. Therefore, more systematic experiments to assess this repair mechanism are currently in progress. In conclusion, we successfully induced the repair of large osteochondral defects with 3-D cartilaginous aggregates formed by bone marrow-derived cells in the RWV bioreactor. This novel technique may have the advantage of achieving a rapid regeneration of cartilage in large osteochondral defects and is a promising technology for the tissue engineering of articular cartilage. Experiments aimed at combining this technique with the use of scaffolds are currently underway. Cartilage tissue of various shapes and sizes could potentially be formed in the RWV bioreactor using scaffolds. Clinical applications for the treatment of focal defects of articular cartilage due to disease or trauma are anticipated in the near future.

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【*Figure legend*】

Figure 1.

(A) A photograph of an aggregate of cartilage formed in a RWV bioreactor.

(B) A histological section of the aggregate. (Safranin-O staining) bar=500 μ m

Figure 2.

Macroscopic appearance of the reparative tissue in Group-C (A,B,C) and Group-T (D,E,F).

(A,D): At 4 weeks (B,E): At 8 weeks (C,F): At 12 weeks after the operation. bar=5mm

Figure 3.

Histological photomicrographs of reparative tissue in Group-T (A-F) and Group-C (G, H, I).

(Safranin-O staining) (A, D, E): At 4 weeks (B): At 8 weeks (C, F): At 12 weeks after the operation. Photomicrographs showing the results of immunohistochemical staining of type Ⅱ (J) and Ⅰ (K) collagen in Group-T.

(A～C, G～I): bar=1000 μ m (D～F, J, K): bar=100 μ m

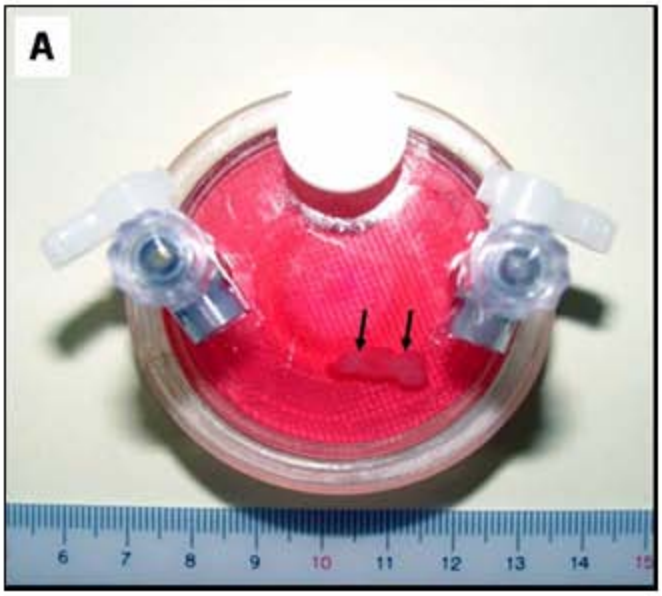
Figure 4.

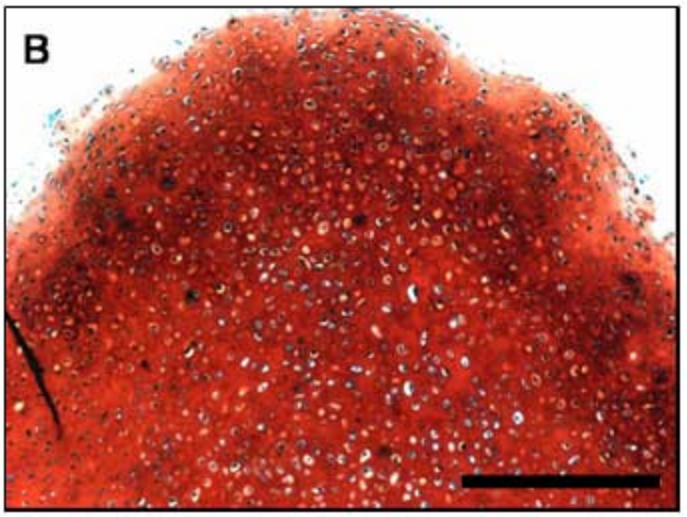
The histological score of the reparative tissue using the O'Driscoll grading scale. (*P＜0.05)

Figure 5.

The time course of the change in the GAG / DNA ratio of the reparative tissue.

Figure 1.





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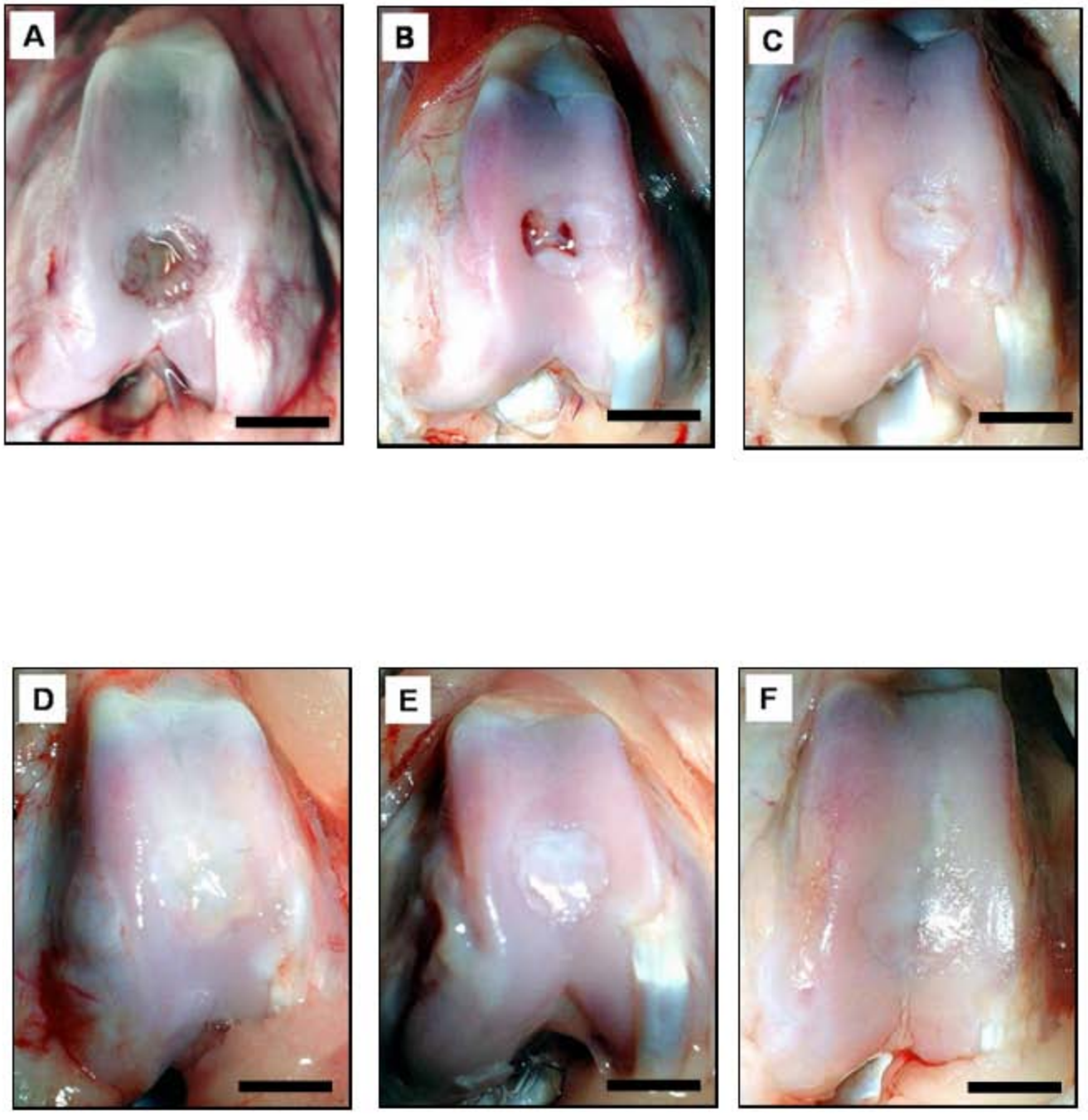
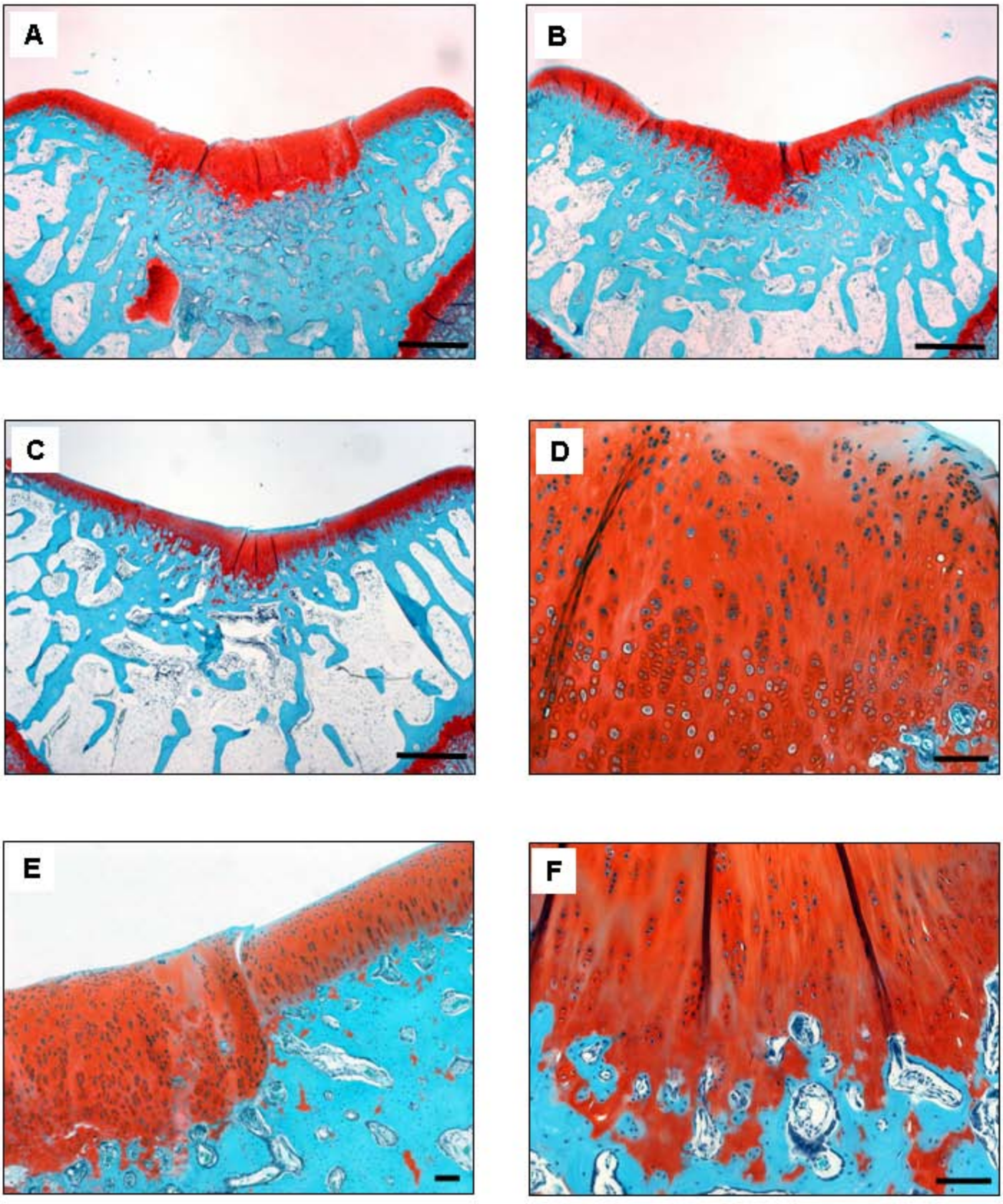
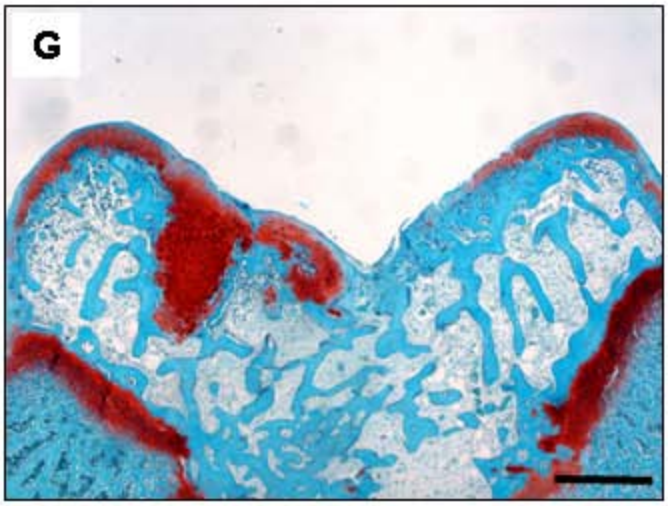


Figure 3.

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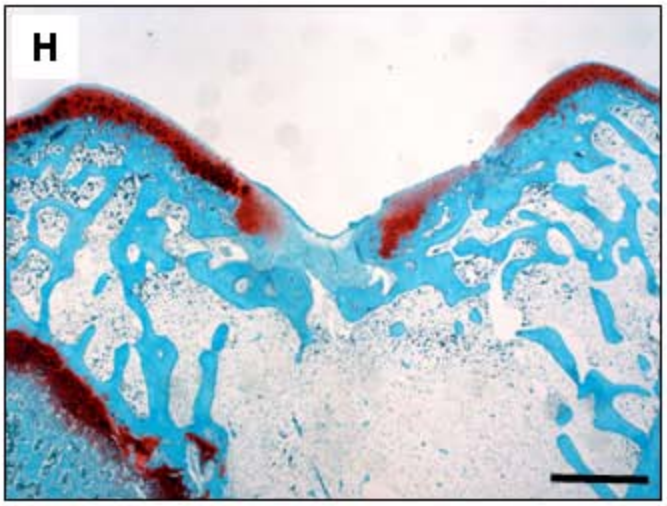
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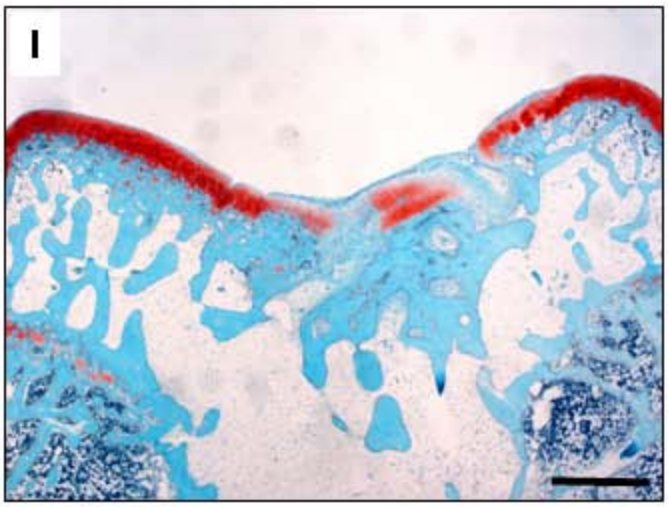
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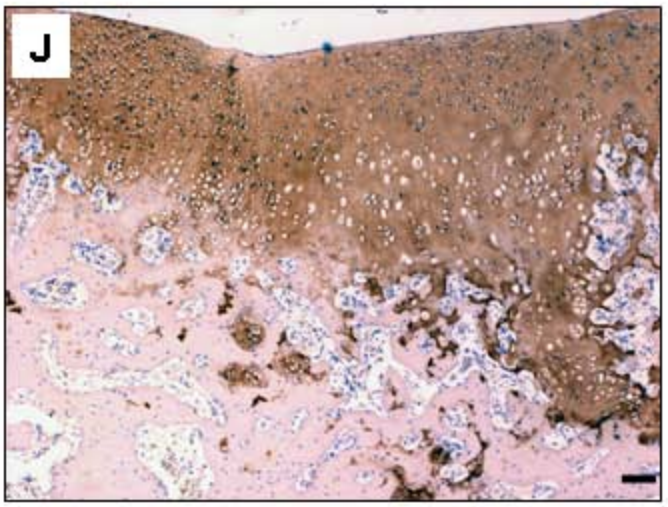
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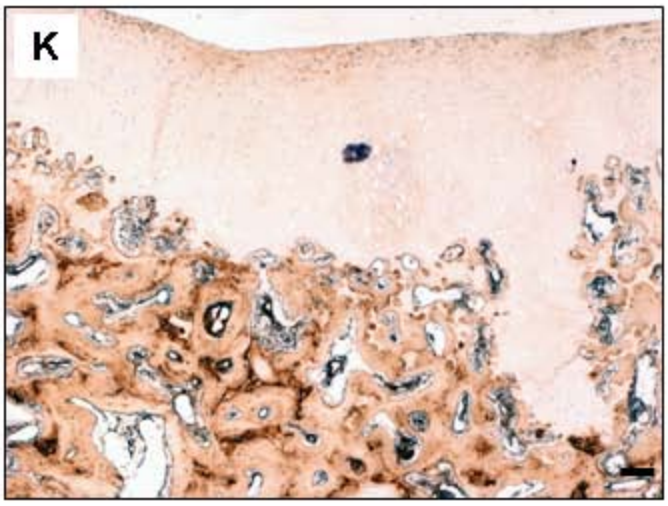
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Figure 4.

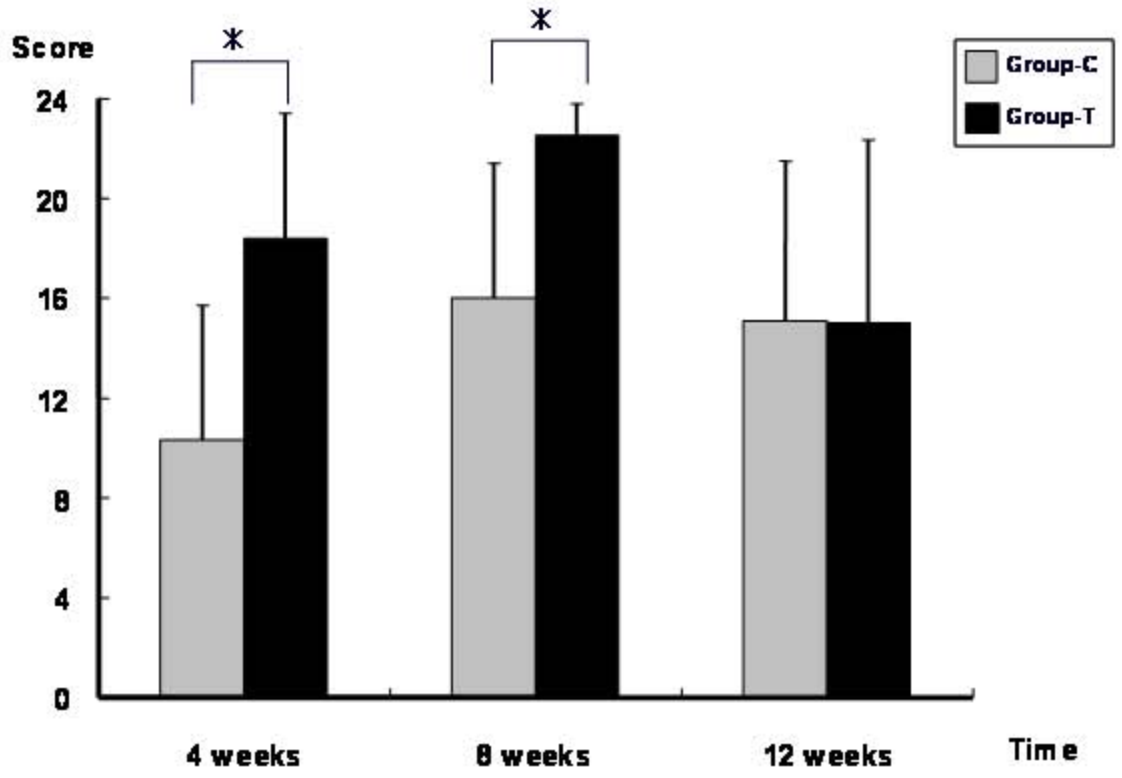
Score
24
20
16
12
8
4
0
*
*
Group-C
Group-T
4 weeks
8 weeks
12 weeks
Time

Figure 5.