



## Repair of articular cartilage defect with layered chondrocyte sheets and cultured synovial cells

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### ABSTRACT

In this study, we investigate the effects of treatment with layered chondrocyte sheets and synovial cell transplantation. An osteochondral defect was created of 48 Japanese white rabbits. In order to determine the effects of treatment, the following 6 groups were produced: (A) synovial cells ( $1.8 \times 10^6$  cells), (B) layered chondrocyte sheets ( $1.7 \times 10^6$  cells), (C) synovial cells ( $3.0 \times 10^5$  cells) + layered chondrocyte sheets, (D) synovial cells ( $6.0 \times 10^5$  cells) + layered chondrocyte sheets, (E) synovial cells ( $1.2 \times 10^6$  cells) + layered chondrocyte sheets, (F) osteochondral defect. Layered chondrocyte sheets and synovial cells were transplanted, sacrificed four and 12 weeks postoperatively. An incapitance tester (Linton) was used to find trends in the weight distribution ratio of the damaged limbs after surgery. Sections were stained with Safranin-O. Repair sites were evaluated using ICRS grading system. In groups (A) to (E), the damaged limb weight distribution ratio had improved. The repair tissue stained positively with Safranin-O. Four and 12 weeks after surgery, groups (A) to (E) exhibited significantly higher scores than group (F), and groups (D) and (E) exhibited significantly higher scores than groups (A) and (B). This suggests the efficacy of combining layered chondrocyte sheets with synovial cells.

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### 1. Introduction

Articular cartilage is avascular tissue nourished by synovial fluid. Articular cartilage shows limited capacity for regeneration after degeneration or injury [1], and leads to osteoarthritis ("OA"). As societies age, much attention is being focused on OA prevention and countermeasures. Treatments for osteochondral defects have included to date: micro fracturing [2–4], mosaicplasty [5–7] and endoprosthetic joint replacement. Beginning with the report by Brittberg et al [11] of autologous chondrocyte implantation (ACI), as a result of development in tissue engineering research a variety of cultured cell graft techniques [11–25] have become the subject of further enquiry. Microfracture surgery and drilling are techniques that encourage natural repair by filling osteochondral defects with marrow-derived repair cells. Normally, an osteochondral defect will induce the production of marrow-derived repair cells [8]. Osteochondral defects are generally thought to be ultimately replaced by subchondral bone after infiltration by blood vessels

during endochondral ossification of chondrocytes from multipotent, marrow-derived MSC [9,10]. Nagai et al. fabricated tissue-engineered cartilage without a scaffold and reported that chondrocyte plates were effective at repairing tissue in animal experiments [21,22]. The usefulness of temperature-responsive culture dishes was reported by Okano et al. [26,27]. Previously, myocardial, corneal and other types of cell sheets have been reported [28–30].

We are continuing to conduct animal experiments with the aim of developing clinical applications for articular cartilage treatment using cell sheets with adhesive properties that were obtained from temperature-responsive culture dishes. Kaneshiro et al. achieved good treatment outcomes by transplantation chondrocyte sheets into partial defect models [31]. Furthermore, Mitani et al. investigated chondrocyte sheets molecular-biologically and immunohistochemically, and examined the chondrocyte repair process [32].

Cartilage repair using synovial cell grafts has been carried out. Hunziker et al have reported synovial cells played an important role in the repair of the cartilage defects [43], and Koga et al have created osteochondral defects in rabbit knee joints and reported good results from grafts of synovium-derived mesenchymal stem cells used in conjunction with periosteum [44]. However, Ando et al investigated repair of articular cartilage using chondrocytes and

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found that the superficial layers of the repaired tissue included fibrous tissue [33]. Further investigation into osteochondral defects using larger animals has been carried out by Ebihara et al using the minipig model and they have previously reported the efficacy of repairs using layered chondrocyte sheets [36]. In order to solve the problem of fibrous tissue being included in the superficial layer in this experiment we investigated the combined use of synovial cells and layered chondrocyte sheet for the repair of articular cartilage.

## 2. Materials and methods

All procedures using animals in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) published by the National Institutes of Health, USA, and the Guidelines of Tokai University on Animal Use.

### 2.1. Temperature-responsive culture dishes

The temperature-responsive culture dishes are coated with Poly(N-isopropylacrylamide) which can change between hydrophilic and hydrophobic depending on the temperature, and was developed by Okano et al. [26,27]. It enables the recovery of sheets of cells without endangering the extracellular matrix, and damage to cells through the use of Trypsin can be avoided. The culture dishes were sterilized using ethylene oxide gas [34]. This product is currently being sold by CellSeed Inc.

### 2.2. Harvesting of chondrocytes and synovial cells from Japanese white rabbits

Four Japanese white rabbits aged 16–18 weeks old and weighing about 3 kg were used as the source of articular chondrocytes and synovial cells. The chondrocytes were harvested from the rabbits' femurs, and the synovial cells were harvested from inside their knee joints. After the cells were enzymatically isolated, the chondrocytes were seeded on temperature-responsive inserts and the synovial cells in temperature-responsive culture dishes, and the cells were cocultured. We understand the need for very long culture times, due to the small amount of chondrocytes capable of being harvested from cartilage and their poor proliferative properties. Huch et al have reported on the interaction between cell types based on a coculture of human synovial cells and chondrocytes [35]. This time, we seeded temperature-responsive inserts with chondrocytes using the method reported by Ebihara, and seeded temperature-responsive culture dishes with synovial cells and cocultured them via the inserts [36]. The results were that there was a significant increase in the activation of proliferation of cells due to the coculture with synovial cells, which made possible the creation of a sheet of chondrocytes in a shorter period of time.

### 2.3. Cell culturing using temperature-responsive culture dishes

The harvested cartilaginous and synovial tissue were finely sliced with scissors and incubated on Petri dishes in DMEM/F12 that contained 0.016% Collagenase Type 1 (Worthington, New Jersey, USA) at 37 °C, 5% CO<sub>2</sub> for 4 h as they were stirred with a stirrer, and the proteins were degraded. Afterwards, the tissue was passed through a cell strainer (BD Falcon™) with a pore size of 100 μm and the cells were retrieved by centrifuge. The chondrocytes were incubated in a culture medium of DMEM/F12 supplemented with 20% fetal bovine serum (FBS; GIBCO, NY, USA) and 1% antibiotics–antimycotic (GIBCO, NY, USA). From day 4 onwards, the culture was maintained by adding a further μg/ml ascorbic acid (Wako Junyakougyou Corp. Japan), and the synovial cells were maintained in a culture medium of DMEM/F12 supplemented with 10% FBS and 1% antibiotics–antimycotic. All culturing was performed at 37 °C, 5% CO<sub>2</sub> and 95% air. The chondrocytes were seeded on temperature-responsive inserts (5.0 cm<sup>2</sup>, CellSeed Inc, Tokyo, Japan) and the synovial cells were seeded in temperature-responsive culture dishes (9.6 cm<sup>2</sup>, CellSeed Inc, Tokyo, Japan) and cocultured for 14 days. Both were seeded at a density of 10,000 cells/cm<sup>2</sup>.

### 2.4. Cell sheet retrieval

After the cells were cultured for two weeks, they reached a confluent state and the temperature-responsive inserts were taken out of the incubator and left for 30 min at 25 °C. After the culture medium was removed, polyvinylidene fluoride (PVDF) membranes were used to retrieve the chondrocyte sheets by the method reported by Yamato et al. [37].

Briefly, the PVDF membrane was placed on the cell sheet and then the sheet was rolled up with the membrane from one corner. This method facilitated good retrieval of the cultured chondrocyte sheets. Next, each retrieved cell sheet was placed on top of a new cell sheet and rolled up in the same way to prepare multilayered sheets. This operation was performed 3 times, and triple-layered chondrocyte sheets were fabricated. Because the layered chondrocyte sheets floated in the culture fluid, cell

strainers (BD Falcon™) were placed on top of them. The layered chondrocyte sheets were cultured for 1 week. The chondrocytes were continuously cultured for one week in temperature-responsive culture dishes.

### 2.5. Transplantation of synovial cells and layered chondrocyte sheets

Forty-eight white Japanese rabbits (female, age: 16–18 weeks, weighing: approximately 3 kg, with each group  $n = 4$ , six groups) were used in this study. For the surgical procedures we used medetomidine (Domitor 1 mg/ml, Meiji Seika Pharma Co., Ltd, Tokyo, Japan) delivered by intramuscular injection.

The rabbits were anesthetized using sevoflurane and O<sub>2</sub> gas. After receiving a medial parapatellar incision to one side leg, the patellae were dislocated laterally and an osteochondral defect (diameter: 5 mm; depth: 3 mm) was created on the patellar groove of the femur using a drill and biopsy punch (REF-BP-50F Kai Industries, Seki, Japan). Bleeding from the bone was observed and osteochondral defects were produced. After two week's incubation, once the cells had become confluent, the temperature-responsive culture dish was removed from the incubator and cooled at 25 °C for 30 min. After separation from the culture substrate, the synovial cells were recovered in pellet form and transplanted onto the osteochondral defect. To obtain further coverage of the defect, layered chondrocytes were grafted onto the defect.

The layered chondrocyte sheets and synovial cells were transplanted under the following 6 conditions: Group (A): synovial cells ( $1.8 \times 10^6$ ) were transplanted, Group (B): only layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted, Group (C): synovial cells ( $3.0 \times 10^5$ ) and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects, Group (D): synovial cells ( $6.0 \times 10^5$ ) and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defect, Group (E): synovial cells ( $1.2 \times 10^6$ ) and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects, Group (F): osteochondral defects only (control group). The synovial cells and layered chondrocyte sheets were transplanted into defects in eight unilateral knees of eight rabbits. After surgery, all of the rabbits were returned to the cage without splinting or immobilization.

### 2.6. Pain evaluation

One day after transplantation, an Incapacitance Tester (Linton Instrumentation, Norfolk, England) was used to find trends in the weight distribution ratio of the undamaged and damaged limbs, and these trends served as the gauge for evaluating pain. The Incapacitance Tester is a device that facilitates automatic and reproducible pain evaluation by measuring (dual channel weight averaging technique) the weight distribution of both hind limbs. This device is widely used to investigate pain ameliorating effects [38]. In order to habituate the animals to the Incapacitance Tester, each day for 7 days after they were delivered, they were all were placed in the main container (holder) of the device and held still for 5 s. The measurements were performed when the animals were still after they were transferred into the rabbit holder, and when they were still after being removed from and then returned to the holder. This operation was conducted 10 times. The weight distribution of both hind legs was measured 10 times, and the following formula was used to calculate the damaged limb weight distribution ratios (%) obtained by loading the left and right limbs.

$$\text{Damaged limb weight distribution ratio (\%)} = \frac{\text{damaged limb load (g)}}{\text{undamaged limb load (g)} + \text{damaged limb load (g)}} \times 100. \quad (1)$$

The average damaged limb weight distribution ratio (%), which was calculated 10 times, was defined as the damaged limb weight distribution ratio (%) per measurement.

After surgery, the measurements were performed a total of 11 times on days 1, 3, 5, 7, 10, 13, 15, 18, 22, 25, and 28.

### 2.7. Histological evaluation of cartilage repair

We used a total of 48 rabbits, with each group  $n = 4$ , six groups sacrificed at 4 weeks, and another six at 12 weeks. Rabbits were sacrificed by an overdose of intravenous anesthetic. The results were evaluated and reviewed. The transplanted tissue was removed from the distal portions of the unilateral femurs. It was then fixed in 4% paraformaldehyde for one week. Afterwards, it was decalcified for 2–3 weeks using distilled water (pH: 7.4) containing 10% ethylenediaminetetraacetic acid (EDTA). Next, the tissue was embedded in paraffin wax and sectioned perpendicular (8 μm sections) through the center of the defect. Each section was stained with safranin O for glycosaminoglycans for histological evaluation.

Immunostaining was performed by the same method as used previously [21,39]. Briefly, deparaffinization was performed using the standard procedure to immunostain the sections. The sections were treated with 0.005% proteinase (type XXIV; Sigma–Aldrich Company, St. Louis, MO, USA) at 37 °C for 30 min. After the sections were washed in PBS (phosphate-buffered saline), they were treated with 0.3% hydrogen peroxide/methanol solution at room temperature for 15–20 min and

endogenous peroxidase was activated. After the sections were washed in PBS, they were reacted for 30 min in a solution containing normal goat serum that had been diluted with PBS at dilution 1:20. Mouse primary monoclonal antibodies, which react with human type I and type II collagen (Daiichi Fine Chemical, Toyama, Japan), were then diluted with PBS containing 1% bovine serum albumin (BSA; Sigma) at dilution 1:200. The sections were left in the solution at 4 °C for one night, then washed 10 times with PBS and reacted at room temperature for 1 h with goat anti-mouse biotin conjugated secondary antibodies that had been diluted with 1% BSA/PBS at dilution 1:100. Afterwards, the sections were treated for 1 h with horseradish peroxidase and dyed with streptavidin (streptavidin HRP). Finally, they were immersed for 2–4 min in Tris–HCl buffer (pH: 7.6) containing 0.05% diaminobenzidine (DAB) and 0.005% hydrogen peroxide. After immunostaining, the slides were counterstained with Mayer's hematoxylin to increase cell visibility.

In the histological evaluation, scoring was carried out by three single blind examiners, using a modified form of Safranin O as reported by O'Driscoll, Keeley and Salter et al [40], and the International Cartilage Repair Society (ICRS) grading system [24,41].

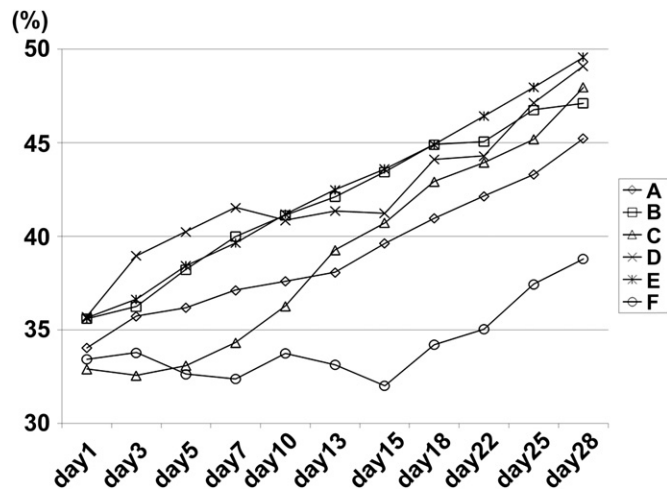
## 2.8. Statistical analysis

The analysis of variance (ANOVA) test was used to analyze the rate of loading 28 days after surgery and the histologic appraisals that were performed using the International Cartilage Repair Society (ICRS) grading system. Fisher's test was used for post hoc testing. The results were expressed as the mean  $\pm$  standard deviation (SD), and  $p < 0.05$  was deemed to be a statistically significant difference.

## 3. Results

### 3.1. Rate of loading trends

Fig. 1 shows the damaged limb weight distribution ratio (mean  $\pm$  SD) on days 1, 3, 5, 7, 10, 13, 15, 18, 22, 25 and 28 after graft surgery. Groups (A) to (E) all exhibited an improvement in the damaged limb weight distribution ratio on day 28 compared to immediately after surgery. The results were as follows: Group (A):  $34.0 \pm 1.6\%$  to  $45.2 \pm 0.7\%$ ; Group (B):  $35.6 \pm 1.7\%$  to  $47.1 \pm 0.6\%$ ; Group (C):  $32.9 \pm 3.4\%$  to  $48.0 \pm 0.3\%$ ; Group (D):  $35.7 \pm 10.1\%$  to



**Fig. 1.** Damaged limb weight distribution ratio (%) after surgery. Damaged limb weight distribution ratio (%) = (damaged limb load (g)/undamaged limb load (g) + damaged limb load (g))  $\times$  100. The damaged limb weight distribution ratio (mean  $\pm$  SD) on days 1, 3, 5, 7, 10, 13, 15, 18, 22, 25 and 28 after surgery is shown. Group (A), immediately after surgery, the damaged limb weight distribution ratio of rabbits that received  $1.8 \times 10^6$  synovial cell transplantation was  $34.0 \pm 1.6\%$ . However, on day 28 this had improved to  $45.2 \pm 0.7\%$ . Group (B), the layered chondrocyte sheets ( $1.7 \times 10^6$  cells) transplantation Group improved from  $35.6 \pm 1.7\%$  to  $47.1 \pm 0.6\%$ . Group (C), the synovial cell ( $3.0 \times 10^5$  cells) and layered chondrocyte sheets transplantation Group improved from  $32.9 \pm 3.4\%$  to  $48.0 \pm 0.3\%$ . Group (D), the synovial cell ( $6.0 \times 10^5$  cells) and layered chondrocyte sheets transplantation Group improved from  $35.7 \pm 10.1\%$  to  $49.1 \pm 1.0\%$ . Group (E), the synovial cell ( $1.2 \times 10^6$  cells) and layered chondrocyte sheets transplantation group improved from  $35.6 \pm 1.1\%$  to  $49.6 \pm 0.1\%$ . Conversely, osteochondral defect Group (F) exhibited poor damaged limb weight distribution ratio improvement of  $33.4 \pm 2.9\%$  to  $38.8 \pm 4.0\%$ .

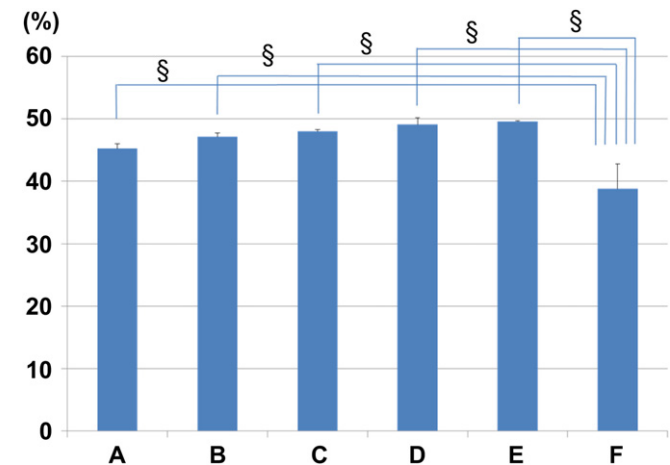
$49.1 \pm 1.0\%$ ; Group (E):  $35.6 \pm 1.1\%$  to  $49.6 \pm 0.1\%$ . Conversely, Group (F) exhibited poor damaged limb weight distribution ratio improvement:  $33.4 \pm 2.9\%$  to  $38.8 \pm 4.0\%$ . Fig. 2 shows the damaged limb weight distribution ratios on day 28 after surgery. Comparative testing with the ANOVA Test revealed significant differences between Groups (A) to (E) and Group (F).

### 3.2. Histological evaluation of repair tissue

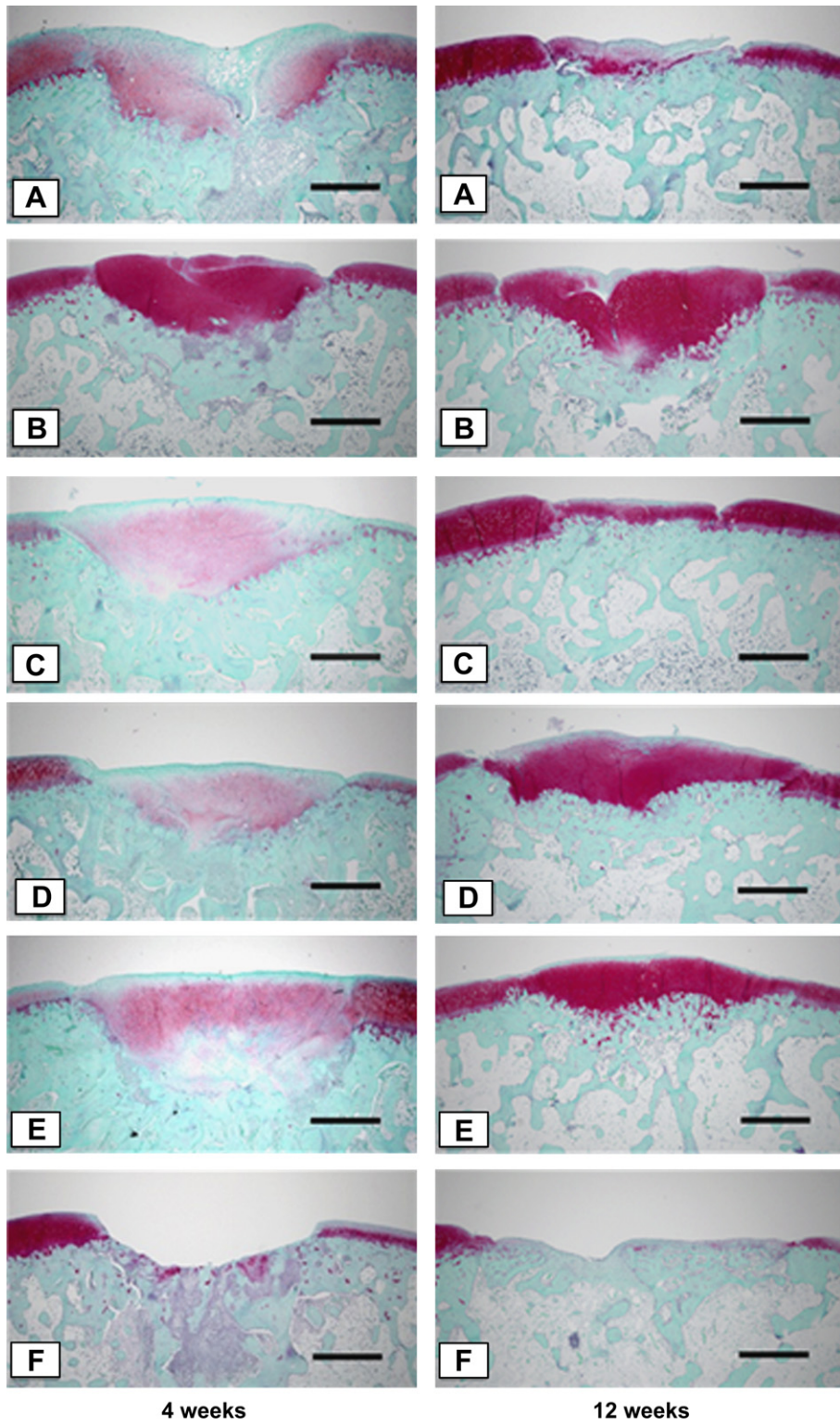
Operations were uneventful. After surgery, all of the rabbits were returned to the cage and allowed to act freely. We did not find any signs of infection. Four and 12 weeks after surgery, four knees from each group were evaluated.

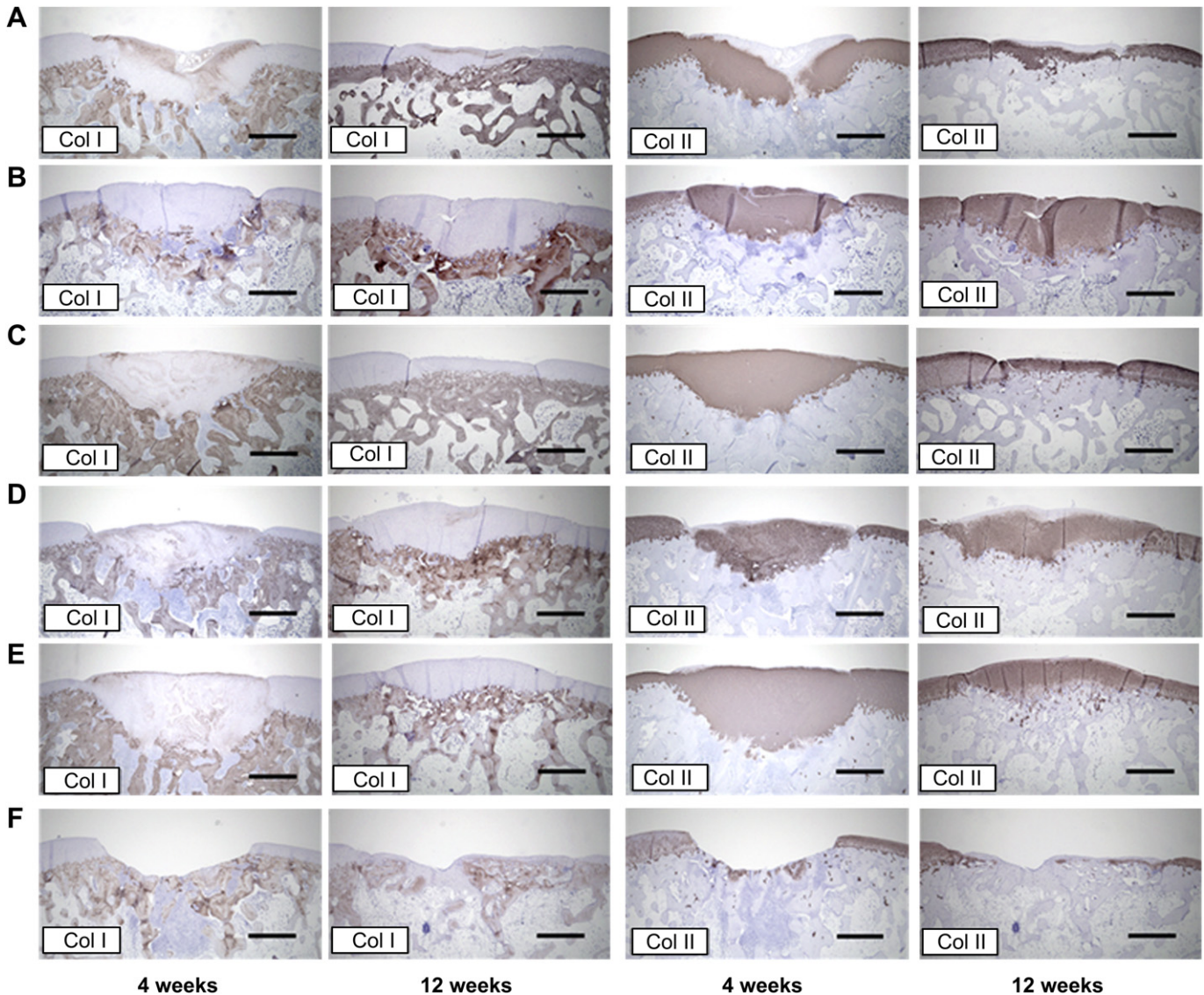
Fig. 3 shows a histological image of repair tissue that was stained with Safranin-O four and 12 weeks after surgery. Four weeks after surgery, the defects of Groups (A) to (E), the cell graft Groups, had been filled with cartilage-like repair tissue. However, we observed that in Group (F), some defects had not been filled with repair tissue. In Group (A), we observed that the implants had been partially replaced with fibrous tissue. Integration with the surrounding cartilage was good. At that time, formation of subchondral bone, including hypertrophic chondrocytes, was inadequate in the lower portion of the implants. In Group (B), Safranin-O staining revealed irregular thickness of the superficial cartilage layer. Integration with the surrounding cartilage was good. Subchondral bone formation was inadequate. In Group (C), Safranin-O staining revealed that integration with surrounding normal cartilage was also good. Although the superficial layer also included some hypertrophic chondrocytes, smooth convex repair was achieved. Similarly, in the case of Groups (D) and (E), Safranin-O staining revealed convex repair tissue formation, and structural consistency, defect filling rates and the condition of the superficial layer of the defect also tended to be better than the other Groups.

Even 12 weeks after transplantation surgery, the defects of Groups (A) to (E), the cell transplantation Groups, had been filled with cartilage-like repair tissue. No cartilage layer was observed in



**Fig. 2.** At 28 days after surgery, a damaged limb weight distribution ratio of ( $p < 0.05$ , §) was deemed significant. VS Group (F). The damaged limb weight distribution ratio on day 28 after surgery is shown. Group (A), the damaged limb weight distribution ratio of rabbits that received  $1.8 \times 10^6$  synovial cell transplantation was  $45.2 \pm 0.7\%$ . Group (B), the layered chondrocyte sheets ( $1.7 \times 10^6$  cells) transplantation Group:  $47.1 \pm 0.6\%$ . Group (C), the synovial cell ( $3.0 \times 10^5$  cells) and layered chondrocyte sheets transplantation Group:  $48.0 \pm 0.3\%$ . Group (D), the synovial cell ( $6.0 \times 10^5$  cells) and layered chondrocyte sheets transplantation Group:  $49.1 \pm 1.0\%$ . Group (E), the synovial cell ( $1.2 \times 10^6$  cells) and layered chondrocyte sheets transplantation Group:  $49.6 \pm 0.1\%$ . Group (F), the osteochondral defect Group:  $38.8 \pm 4.0\%$ . Significant differences were observed between Groups (A) to (E) and Group (F).





**Fig. 4.** Histological picture of repair tissue that was immunostained four and 12 weeks after surgery. (Bar = 1000  $\mu$ m). Group (A):  $1.8 \times 10^6$  synovial cells were transplanted. Group (B): only layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted. Group (C):  $3.0 \times 10^5$  synovial cells and layered chondrocyte sheets  $1.7 \times 10^6$  synovial cells were transplanted on top to cover the osteochondral defects. Group (D):  $6.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (E):  $1.2 \times 10^6$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (F): osteochondral defects only (control Group). Immunostaining was performed with type I (Col I) and type II (Col II) collagen, and the results were evaluated and reviewed. Four weeks after surgery, in Groups (A) to (E), type II collagen expression was observed in implant tissue that had been stained with Safranin-O, it was expressed uniformly in the surrounding cells. Type II collagen made the cartilaginous repair tissue borders clearer. Conversely, although type I collagen expression was not observed in the portions that had been stained with Safranin-O, it was observed in the superficial portion and superficial layer of subchondral bone that had been replaced with fibrous tissue. Twelve weeks after surgery, similarly, in Groups (A) to (E), type II collagen expression was observed in the implant tissue and pericellular normal cartilage, and type I collagen expression was observed in the superficial portion of fibrocartilage and the superficial layer of subchondral bone.

**Fig. 3.** Histologic findings for repair tissue that was stained with Safranin-O four and 12 weeks after surgery (Bar = 1000  $\mu$ m). In Group (A), Safranin-O staining was performed on the group that received  $1.8 \times 10^6$  synovial cell transplantation. Four weeks after surgery, however, we observed that the implants had been partially replaced with fibrous tissue. At that time, formation of subchondral bone, including hypertrophic chondrocytes, was inadequate in the lower portion of the graft tissue. Twelve weeks after surgery, the defect had been filled with cartilage-like repair tissue. Although fibrous tissue was observed in the superficial portion, subchondral bone formation was good. In Group (B), four weeks after surgery, Safranin-O staining performed on the Group that received layered chondrocyte sheets ( $1.7 \times 10^6$  cells) transplantation revealed that integration with the surrounding cartilage was good. Subchondral bone formation was inadequate. Twelve weeks after surgery, fibrous tissue was observed in portions of the superficial layer of repair tissue that were stained with Safranin-O. However, defect filling rates, subchondral bone and integration with the surrounding cartilage layer were good. In Group (C), synovial cells ( $3.0 \times 10^5$ ) and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) transplanted on top to cover the osteochondral defects. Four weeks after surgery, integration with the surrounding normal cartilage was good. Although the superficial layer also included some hypertrophic chondrocytes, smooth convex repair was achieved. Twelve weeks after surgery, fibrous tissue was observed in the superficial portion; however, Safranin-O stainability and subchondral bone formation were good. In Group (D), synovial cells ( $6.0 \times 10^5$ ) and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) transplanted on top to cover the osteochondral defects. Four weeks after surgery, the formation of convex repair tissue was achieved, and structural consistency, defect filling rates and condition of the superficial layer of the defect were good. Twelve weeks after surgery, the cartilage layer exhibited a columnar arrangement, and good repair was achieved. In Group (E),  $1.2 \times 10^6$  cells and layered chondrocyte sheets ( $1.7 \times 10^6$ ) transplanted on top to cover the osteochondral defects. Four weeks after surgery, similarly, the formation of convex repair tissue (stained with Safranin-O) was achieved, and structural consistency, defect filling rates and condition of the superficial layer of the defect were good. Twelve weeks after surgery, similarly, the condition of the implants tissue, tissue filling rates and subchondral bone formation were all good, and the transplant cartilage layer exhibited a columnar arrangement and had been repaired with hyaline cartilage that appeared almost normal. In Group (F), osteochondral defects only (control Group). Four weeks after surgery, we observed that the defects had not been filled with repair tissue. Twelve weeks after surgery, the implant tissue exhibited no cartilage layer, and had not been replaced with bone.

the repair tissue of Group (F), and the defect had been replaced with bone. Fibrous tissue was observed in the superficial portion of Group (A) but subchondral bone formation was good. In the case of Group (B), fibrous tissue was observed in parts of the superficial layer of repair tissue that had been stained with Safranin-O; however, defect filling rates, subchondral bone and integration with the surrounding cartilage layer were good. Fibrous tissue was also observed in parts of the superficial layer of Group (C); however, Safranin-O stainability and subchondral bone formation were good. In Group (D), the cartilage layer exhibited a columnar arrangement, and good repair was achieved. In Group (E), the condition of the graft tissue, defect filling rates and subchondral bone formation were all good. The graft cartilage layer exhibited a columnar arrangement, and had been repaired with hyaline cartilage that appeared almost normal. In Group (F), no cartilage layer had formed in the transplantation tissue, and the defect had been replaced with bone.

### 3.3. Immunohistochemical evaluation

Fig. 4 shows a histological picture of repair tissue that was immunostained four and 12 weeks after surgery. Four weeks after surgery, in Groups (A) to (E), we observed type II collagen expression in tissue that had been stained with Safranin-O. Type II collagen was expressed uniformly in the surrounding cells. In Group (A), no type II collagen expression was observed in the portion of the defect that had been replaced by fibrous tissue. Type II collagen made the cartilaginous repair tissue borders clearer. Conversely, although type I collagen expression was not observed

in the portions that had been stained with Safranin-O, it was observed in the superficial portion and superficial layer of subchondral bone that had been replaced with fibrous tissue.

Similarly, 12 weeks after surgery, type II collagen expression was observed in the grafted tissue and pericellular normal cartilage of groups (A) to (E), and type I collagen expression was observed in the superficial portion of fibrocartilage and the superficial layer of subchondral bone.

### 3.4. Histological scoring of repair tissue

We evaluated the repair tissue using the ICRS histological grading system [24,40,41] (Table 1), which is a modification of the grading system developed by O'Driscoll, Keeley and Salter. This system evaluates repair tissue based on 11 items: tissue morphology (Ti); matrix staining (Matx); structural integrity (Stru); cluster formation (Clus); tidemark opening (Tide); bone formation (Bform); histologic appraisal of surface architecture (SurfH); histologic appraisal of the degree of defect filling (FilH); lateral integration of defect-filling tissue (LatI); basal integration of defect-filling tissue (BasI); and histologic signs of inflammation (InfH). The total scores range ranged from 11 to 45. Table 2 and Fig. 5 show the ICRS grading system results four weeks after surgery. Four weeks after surgery, the results were as follows: Group (A):  $26.8 \pm 3.8$ ; Group (B):  $25.0 \pm 6.2$ ; Group (C):  $30.5 \pm 3.8$ ; Group (D):  $35.0 \pm 4.2$ ; Group (E):  $35.8 \pm 3.8$ ; Group (F):  $17.0 \pm 1.2$ . Groups (A) to (E) exhibited significantly higher scores than Group (F). Significant differences were also observed between Group (A) and Groups (D) and (E), and Group (B) and Groups (D) and (E). Viewed by item,

**Table 1**

ICRS Histological grading system. This system evaluates repair tissue based on 11 items: tissue morphology (Ti); matrix staining (Matx); structural integrity (Stru); cluster formation (Clus); tidemark opening (Tide); bone formation (Bform); histologic appraisal of surface architecture (SurfH); histologic appraisal of the degree of defect filling (FilH); lateral integration of defect-filling tissue (LatI); basal integration of defect-filling tissue (BasI); and histologic signs of inflammation (InfH). The total scores range ranged from 11 to 45.

<b>Ti</b>	<b>Tide</b>	<b>LatI</b>
Tissue morphology	Intactness of the calcified cartilage layer, formation of tidemark	Lateral integration of implanted material
4: mostly hyaline cartilage	1: <25% of the calcified cartilage layer intact	1: not bonded
3: mostly fibrocartilage	2: 25–49% of the calcified cartilage layer intact	2: bonded at one end/partially both ends
2: mostly non-cartilage	3: 50–75% of the calcified cartilage layer intact	3: bonded at both sides
1: exclusively non-cartilage	4: 76–90% of the calcified cartilage layer intact	
	5: complete intactness of the calcified cartilage layer	
<b>Matx</b>	<b>Bform</b>	<b>BasI</b>
Matrix staining	Subchondral bone formation	Basal integration of implanted material
1: none	1: no formation	1: <50%
2: slight	2: slight	2: 50–70%
3: moderate	3: strong	3: 70–90%
4: strong		4: 91–100%
<b>Stru</b>	<b>SurfH</b>	InfH
Structural integrity	Histologic appraisal of surface architecture	Inflammation
1: severe disintegration	1: severe fibrillation or disruption	1: no inflammation
2: cysts or disruptions	2: moderate fibrillation or irregularity	3: slight inflammation
3: no organization of chondrocytes	3: slight fibrillation or irregularity	5: strong inflammation
4: beginning of columnar organization of chondrocytes	4: normal	
5: normal, similar to healthy mature cartilage		
<b>Clus</b>	<b>FilH</b>	<b>Hgtot</b>
Chondrocyte clustering in implant	Histologic appraisal defect filling	Histological grading system
1: 25–100% of the cells clustered	1: <25%	Some of the histologic variables:
2: <25% of the cells clustered	2: 26–50%	tissue morphology (Ti),
3: no clusters	3: 51–75%	matrix staining (Matx), structural integrity (Stru),
	4: 76–90%	cluster formation (Clus), tidemark opening (Tide),
	5: 91–110%	bone formation (Bform), histologic surface architecture (SurfH),
		histologic degree of defect filling (FilH),
		lateral integration of defect-filling tissue (LatI),
		basal integration of defect-filling tissue (BasI),
		and histologic signs of inflammation (InfH)
		Maximum total: 45 points

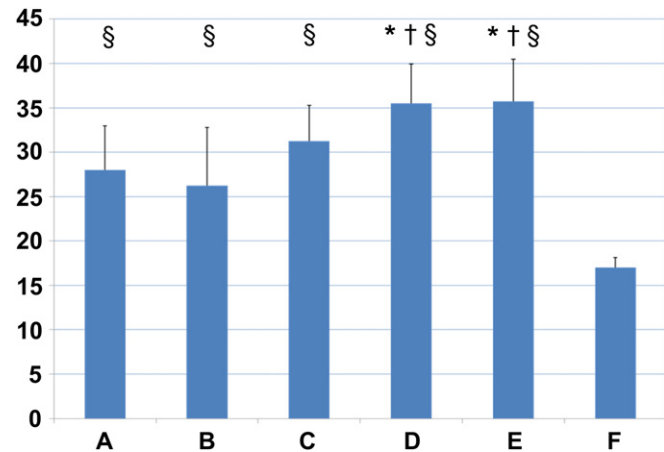
**Table 2**

ICRS grading system 4W; values are the mean ± SD. The total score range is from 11 (no repair) to 45 (normal articular cartilage). Group (A):  $1.8 \times 10^6$  synovial cells were transplanted. Group (B): only layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted. Group (C):  $3.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (D):  $6.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (E):  $1.2 \times 10^6$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (F): osteochondral defects only (control group). Four weeks after graft surgery, histologic appraisals were performed using the ICRS grading system. The results were as follows: Group (A):  $26.8 \pm 3.8$ ; Group (B):  $25.0 \pm 6.2$ ; Group (C):  $30.5 \pm 3.8$ ; Group (D):  $35.0 \pm 4.2$ ; Group (E):  $35.8 \pm 3.8$ ; Group (F):  $17.0 \pm 1.2$ . Viewed by item, Groups (A) to (E) exhibited significantly higher Matx, FilH, and Basl scores than Group (F), while Groups (D) and (E) exhibited significantly higher Clus and Tide scores than Groups (A) and (B).

	A	B	C	D	E	F
Ti	2.50 ± 0.6	2.25 ± 0.5	3.00 ± 0.0	3.00 ± 0.8	3.25 ± 0.5	1.50 ± 0.6
Matx	1.25 ± 0.5	2.00 ± 0.8	2.50 ± 0.6	3.00 ± 0.8	2.75 ± 0.5	1.00 ± 0.0
Stru	2.75 ± 1.0	2.50 ± 1.0	3.50 ± 0.6	3.75 ± 0.5	3.75 ± 0.5	2.00 ± 0.0
Clus	1.50 ± 0.6	1.25 ± 0.5	1.50 ± 0.6	2.00 ± 0.0	2.00 ± 0.0	1.00 ± 0.0
Tide	1.25 ± 0.5	1.50 ± 1.0	2.00 ± 0.8	3.25 ± 0.5	3.50 ± 1.0	1.00 ± 0.0
Bform	1.75 ± 0.5	2.00 ± 0.0	2.25 ± 0.5	2.25 ± 0.5	2.50 ± 0.6	1.50 ± 0.6
SurfH	1.75 ± 0.6	2.00 ± 0.8	1.50 ± 0.6	2.25 ± 0.5	2.25 ± 0.5	1.00 ± 0.0
FilH	3.00 ± 0.8	2.25 ± 1.3	3.50 ± 1.3	4.00 ± 0.8	4.00 ± 1.4	1.00 ± 0.0
Latl	2.50 ± 1.0	1.50 ± 0.6	2.25 ± 0.5	2.75 ± 0.5	2.75 ± 0.5	1.00 ± 0.0
Basl	3.50 ± 0.6	2.75 ± 0.5	3.50 ± 0.6	3.75 ± 0.5	4.00 ± 0.0	1.00 ± 0.0
InfH	5.00 ± 0.0	5.00 ± 0.0	5.00 ± 0.0	5.00 ± 0.0	5.00 ± 0.0	5.00 ± 0.0
Hgtot	26.8 ± 3.8	25.0 ± 6.2	30.5 ± 3.8	35.0 ± 4.2	35.8 ± 3.8	17.0 ± 1.2

Groups (A) to (E) exhibited significantly higher Matx, FilH, and Basl scores than Group (F), while Groups (D) and (E) exhibited significantly higher Clus and Tide scores than Group (B).

Table 3 and Fig. 6 show the ICRS grading system results 12 weeks after surgery. Group (A):  $29.0 \pm 0.8$ ; Group (B) Group:  $31.8 \pm 5.4$ ; Group (C):  $32.3 \pm 5.0$ ; Group (D):  $38.8 \pm 2.1$ ; Group (E):  $40.8 \pm 2.5$ ; Group (F):  $23.3 \pm 2.4$ . Similarly, four weeks after surgery, Groups (A) to (E) exhibited significantly higher scores than Group



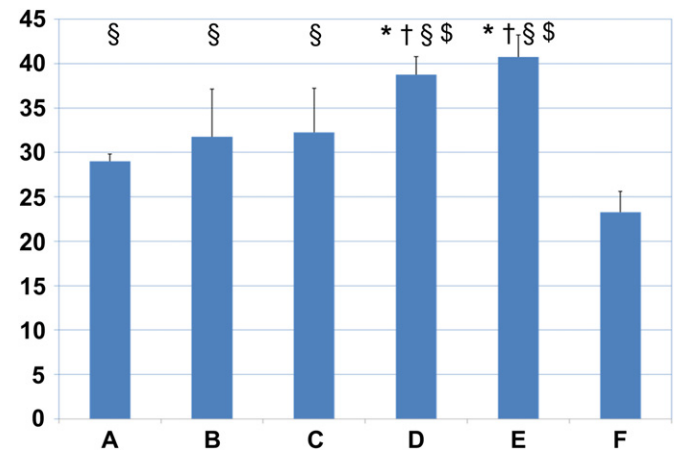
**Fig. 5.** ICRS grading system 4W, ( $p < 0.05$ ) was deemed significant. (§) VS Group (F), (\*) VS Group (A), (†) VS Group (B). Group (A):  $1.8 \times 10^6$  synovial cells were transplanted. Group (B): only layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted. Group (C):  $3.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (D):  $6.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (E):  $1.2 \times 10^6$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (F): osteochondral defects only (control group). Four weeks after surgery, histologic appraisals were performed using the ICRS grading system. Groups (A) to (E) exhibited significantly higher scores than Group (F). Significant differences were observed between Group (A) and Groups (D) and (E), and Group (B) and Groups (D) and (E).

**Table 3**

ICRS grading system 12W, Values are the mean ± SD. The total score range is from 11 (no repair) to 45 (normal articular cartilage). Group (A):  $1.8 \times 10^6$  synovial cells were transplanted. Group (B): only layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted. Group (C):  $3.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (D):  $6.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were on top to cover the osteochondral defects. Group (E):  $1.2 \times 10^6$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (F): osteochondral defects only (control group). Twelve weeks after surgery, histologic appraisals were performed using the ICRS grading system. Group (A):  $29.0 \pm 0.8$ ; Group (B):  $31.8 \pm 5.4$ ; Group (C):  $32.3 \pm 5.0$ ; Group (D):  $38.8 \pm 2.1$ ; Group (E):  $40.1 \pm 2.5$ ; Group (F):  $23.3 \pm 2.4$ . Viewed by item, Groups (A) to (E) exhibited significantly higher Matx and InfH scores than Group (F), while Groups (D) and (E) exhibited significantly higher SurfH, FilH and Tide scores than Groups (A) and (B).

	A	B	C	D	E	F
Ti	2.00 ± 0.8	2.50 ± 1.3	2.75 ± 1.5	3.25 ± 1.0	3.75 ± 0.5	1.75 ± 0.5
Matx	2.50 ± 0.6	2.25 ± 0.5	2.50 ± 0.6	3.25 ± 0.5	3.50 ± 0.6	1.25 ± 0.5
Stru	2.50 ± 0.6	3.25 ± 1.0	3.25 ± 1.0	3.75 ± 0.5	4.25 ± 0.5	2.25 ± 0.5
Clus	2.00 ± 0.0	3.00 ± 0.0	2.75 ± 0.5	3.00 ± 0.0	3.00 ± 0.0	1.75 ± 0.5
Tide	2.25 ± 0.5	2.00 ± 0.8	2.00 ± 1.4	3.75 ± 0.5	4.25 ± 0.5	1.25 ± 0.5
Bform	2.25 ± 0.5	1.75 ± 1.0	2.00 ± 1.2	3.00 ± 0.0	2.25 ± 0.5	1.75 ± 1.0
SurfH	2.00 ± 0.0	1.50 ± 0.6	2.25 ± 0.5	2.75 ± 0.5	2.75 ± 0.5	1.00 ± 0.0
FilH	3.25 ± 0.5	3.75 ± 0.5	4.25 ± 0.5	4.50 ± 0.6	5.00 ± 0.0	3.50 ± 0.6
Latl	2.25 ± 0.5	2.75 ± 0.5	2.25 ± 1.0	2.50 ± 0.6	3.00 ± 0.0	1.75 ± 1.0
Basl	3.50 ± 0.6	4.00 ± 0.0	3.25 ± 1.0	4.00 ± 0.0	4.00 ± 0.0	3.50 ± 0.6
InfH	4.50 ± 1.0	5.00 ± 0.0	5.00 ± 0.0	5.00 ± 0.0	5.00 ± 0.0	3.50 ± 1.0
Hgtot	29.0 ± 0.8	31.8 ± 5.4	32.3 ± 5.0	38.8 ± 2.1	40.8 ± 2.5	23.3 ± 2.4

(F), and significant differences were observed between Group (A) and Groups (D) and (E) and Group (B) and Groups (D) and (E). At 12 weeks, significant differences were also observed between Group (C) and Groups (D) and (E). Viewed by item, Groups (A) to (E) exhibited significantly higher Matx and InfH scores than Group (F), while Groups (D) and (E) exhibited significantly higher SurfH, FilH and Tide scores than Groups (A) and (B).



**Fig. 6.** ICRS grading system 12W, ( $P < 0.05$ ) was deemed significant. (§) VS Group (F), (\*) VS Group (A), (†) VS Group (B), (§) VS Group (C). Group (A):  $1.8 \times 10^6$  synovial cells were transplanted. Group (B): only layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted. Group (C):  $3.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (D):  $6.0 \times 10^5$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (E):  $1.2 \times 10^6$  synovial cells and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) were transplanted on top to cover the osteochondral defects. Group (F): osteochondral defects only (control group). Twelve weeks after surgery, histologic appraisals were performed using the ICRS grading system. Similarly, four weeks after surgery, Groups (A) to (E) exhibited significantly higher scores than Group (F), and significant differences were observed between Group (A) and Groups (D) and (E), and Group (B) and Groups (D) and (E). At 12 weeks, significant differences were also observed between Group (C) and Groups (D) and (E).

#### 4. Discussion

In recent years, there has been widespread use of cell grafts to repair articular cartilage, both in animal experiments [15,17,18,21–25] and in clinical application [11–14,16,19,20]. Ochi et al have reported that better results have been obtained using grafts of tissue-engineered cartilage than chondrocytes, and have obtained good results in clinical application using tissue-engineered cartilage grafts embedded in atelocollagen gel [42]. Generally speaking, scaffolds consist of synthetic polymers or biological materials, and therefore, there are numerous concerns regarding their long-term biocompatibility [18]. In order to eliminate these unknown risks, it would be ideal not to use scaffolds. From this perspective, scaffold free cell grafts would be an excellent option. Mainil-Varlet P et al. produced scaffold free tissue-engineered cartilage using a static bioreactor system [23]. Nagai et al. produced tissue-engineered cartilage using a rotation culture method and a lower cell count ( $6.0 \times 10^6$  cells) [22]. Synovial cell grafts are widely used to repair articular cartilage defects. Hunziker et al have reported synovial cells played an important role in the repair of the cartilage defects [43]. Koga et al have reported good results from creating osteochondral defects in rabbit knees and then grafting synovium-derived MSCs used in conjunction with periosteum [44]. Ando et al have reported the inclusion of fibrous tissue in repaired tissue after investigating articular cartilage repair using synovial cells [33]. The current study sought to repair articular cartilage through the combined use of layered chondrocyte sheets and synovial cells, aiming for completely hyaline cartilage repair by solving the problem of fibrous tissue inclusion. Previously, Kaneshiro et al. reported that layered chondrocyte sheets have an inhibiting effect on cartilage degeneration using cartilage partial-thickness defect models [31], and Ebihara et al. reported good effects from treatment with layered chondrocyte sheets using minipig osteochondral defect models; however, they reported that in some cases, subchondral bone formation was inadequate [36]. It has been reported that layered chondrocyte sheets may be excellent cell grafts that are scaffold free and possess a three-dimensional structure; in this study, we investigated articular cartilage repair by combining layered chondrocyte sheets with synovial cells in an effort to achieve a better tissue-like structure.

Four weeks after surgery, the transplanted cell groups improved significantly compared to the control groups, which suggests that the cell implants were effective at ameliorating pain. This implies that the synovial cell or chondrocyte sheet implants repaired the articular cartilage defects, improving the defects to the point that they could withstand loading.

In the histologic appraisal, significant differences between groups (A) to (E) and group (F) were observed four and 12 weeks after surgery, suggesting that cell transplantation contribute more to tissue repair than non-cell implants. Furthermore, significant differences were observed between groups (A) and (B) and groups (D) and (E), which suggests that the combination of layered chondrocyte sheets with synovial cell transplantation was effective.

Ando et al. investigated articular cartilage repair using synovial cell implants and found that the superficial portion contained fibrous tissue. Therefore, they reported that synovial cell implants might promote chondrogenic differentiation *in vivo* [33]. From the perspective of implant cell counts, the synovial cell counts we employed (group (A):  $1.8 \times 10^6$  cells (C):  $3.0 \times 10^5$  cells (D):  $6.0 \times 10^5$  cells (E):  $1.2 \times 10^6$  cells) were clearly lower than ACI reports [44] which used other synovial cells.

In this study, we achieved good repair by combining synovial cell transplantation with layered chondrocyte sheets, even though only few synovial cell implants were used. Nevertheless, although no significant differences between group (A) and groups (B) and (C)

or group (C) and groups (D) and (E) were observed four weeks after surgery, significant differences between group (C) and groups (D) and (E) were observed 12 weeks after surgery. These results indicate that if it transplants only synovial cells which may be insufficient, and layered chondrocyte sheets and synovial cells will be combined and transplanted into osteochondral defects, the grafting of  $6.0 \times 10^5$  or more synovial cells may be beneficial. In the future, further study will be required to optimize the implant cell count given factors that include varying states of repair, knee joint volume and osteochondral defect size depending on the animal species. This report suggests that a combination of layered chondrocyte sheets and synovial cells may be effective in repairing articular cartilage.

#### 5. Conclusions

In rabbit osteochondral defect models, pain alleviating effects and better tissue repair were achieved by combining layered chondrocyte sheets with synovial cells transplantations. The condition of Group (E): synovial cells ( $1.2 \times 10^6$ ) and layered chondrocyte sheets ( $1.7 \times 10^6$  cells) demonstrated excellent results of both defect filling rates and subchondral bone formation. The graft cartilage layer exhibited a columnar arrangement, and had been repaired with hyaline cartilage. Transplantation conditions and other factors must therefore be further investigated.

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