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RESEARCH ARTICLE

The effects of using vitrified chondrocyte sheets on pain alleviation and articular cartilage repair

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Abstract

The effect of using vitrified–thawed chondrocyte sheets on articular cartilage repair **was examined** because the methods for storing chondrocyte sheets are essential for allogeneic chondrocyte sheet transplantation. Six Japanese white rabbits were used as sources of articular chondrocytes and synovial cells. Chondrocytes were harvested from the femur, and synovial cells were harvested from inside the knee joints. After coculture of the chondrocytes with synovial cells, triple-layered chondrocyte sheets were fabricated. Eighteen rabbits were used, with six rabbits in each of three groups: osteochondral defect only (control, group A); chondrocyte sheets (group B); and vitrified–thawed chondrocyte sheets (group C). An osteochondral defect was created on the femur. After transplantation, the weight distribution ratio of the undamaged and damaged limbs was measured as a pain-alleviating effect. The rabbits were euthanized at 12 weeks, and the transplanted tissues were evaluated for histology (Safranin O staining and immunostaining) using the International Cartilage Repair Society grading system. For both evaluations, significant differences were observed between groups A and B, and between groups A and C ($p < 0.05$). No significant differences were observed between groups B and C. Thus, pain-alleviating effects and tissue repair were achieved using vitrified–thawed chondrocyte sheets. Copyright © 2017 John Wiley & Sons, Ltd.

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Keywords cartilage regeneration; chondrocyte sheet; cryopreservation; temperature-responsive culture dish; transplantation; vitrification

1. Introduction

Osteoarthritis (OA) is an intractable and slowly progressing disease caused by articular cartilage degeneration. With progression, it causes dysfunction of the knee joint and makes activities of daily living difficult. Posttraumatic articular cartilage defects are typically treated by microfracture (Steadman *et al*., 2002), mosaicplasty (Hangody *et al*., 2001), or autologous chondrocyte implantation (Ochi *et al*., 2002). There are other treatments for OA caused by articular cartilage degeneration and damage, such as high tibial osteotomy and total knee arthroplasty. However, these treatments are not intended to prevent human articular cartilage degeneration and damage (Hunziker and Rosenberg, 1996; Kaneshiro *et al*., 2006). As societies are becoming increasingly aged, there are concerns that the incidences of dysbasia and of bedridden patients with OA of the knee will increase further. Therefore, to maintain the activity of elderly individuals, prolong their healthy life, and reduce expenses for care and medicine, it is necessary to develop basic treatments for this disease.

As a treatment for damaged articular cartilage, a variety of autologous chondrocyte implantations using chondrocytes recovered from patients, then cultured *in vitro* and transplanted to articular cartilage defects, have been performed both within and outside Japan. Posttraumatic articular cartilage defects are the main indications for this treatment but OA is not, unless such untreated cartilage defects progress to it (Ando *et al*., 2007). Using conventional cell therapies, the possibility of exacerbating OA is a concern because fibrous cartilage is mixed in with the regenerated tissue. We have already reported the usefulness of layered chondrocyte sheets as a treatment for degenerated articular cartilage in animal experiments. These include: studies to define suitable scaffolds (e.g., atelocollagen honeycomb-shaped scaffolds with a membrane seal, or chitosan hydrogels) for cartilage regeneration (Ishihara *et al*., 2002; Sato *et al*., 2003); optimizing extracellular environments with growth factors and a rotational culture system (Ishihara *et al*., 2001; Masuoka *et al*., 2005; Nagai *et al*., 2008a); cartilage repair by means of scaffold and chondrocyte implantation (Nagai *et al*., 2008b; Sato *et al*., 2007); and chondrocyte sheet transplantation, as well as checks of the properties of chondrocyte sheets and regenerated cartilage (Kaneshiro *et al*., 2006, 2007; Mitani *et al*., 2009; Sato *et al*., 2008). These studies have confirmed the importance of the extracellular environment in the repair and regeneration of articular cartilage. Moreover, the cells of recipients promote repair proactively if there is a minimum amount of cartilage needed for tissue engineering (Masuoka

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et al., 2005; Nagai *et al.*, 2008b). Kaneshiro *et al.* (2006) reported the effect of layered chondrocyte sheets obtained from temperature-responsive culture dishes as a treatment for partial-thickness defects, which do not reach the subchondral bone, resemble early osteoarthritis, and are considered difficult to deal with conventionally. In addition, the characteristics of humoral factors produced by layered chondrocyte sheets have been clarified, and experiments in humans have shown that synovial cell cocultures provide an optimal environment for the preparation of such sheets for tissue transplantation, and are particularly beneficial for shortening the required culture period (Hamahashi *et al.*, 2015; Kokubo *et al.*, 2013; Mitani *et al.*, 2009). Furthermore, using experiments in rats (Takaku *et al.*, 2014), rabbits (Ito *et al.*, 2012) and minipigs (Ebihara *et al.*, 2012), we achieved good treatment outcomes by transplanting chondrocyte sheets into models of full-thickness defects that reach subchondral bone. These approaches are effective in treating both partial- and full-thickness defects. Studies have suggested that chondrocyte sheets have therapeutic effects in both types of cartilage damage, as there is always a mix of partial-thickness defects (damage confined to cartilage) and full-thickness defects (damage reaching the subchondral bone) in cases of OA, and so chondrocyte sheets are promising options for cartilage regeneration. As a feature of this treatment, repair with hyaline cartilage – impossible with conventional chondrocyte transplantation – can be achieved because chondrocytes only cover the surface of the defect, which is repaired by the differentiation of mesenchymal stem cells induced by bone marrow stimulation into chondrocytes. Surface sheets of chondrocytes prevent erosion of the cartilaginous matrix. They also block the escape of bone marrow cells into the synovial fluid, release anabolic factors to the defect, and physically block catabolic factors in synovial fluid. As a result, they promote the differentiation of bone marrow cells while protecting the damaged part, and eventually enable cartilage regeneration. This therapeutic method relies on interactions between recipient and donor cells in the repair and regeneration of articular cartilage. Cartilage regeneration by hyaline cartilage has been achieved in a clinical study currently under way (data not shown). Thus, this treatment can be an effective method for repairing cases of OA in the knee with cartilaginous defects.

Problems associated with autografts include the need for two operations: first to harvest chondrocytes and synovial cells, and then to transplant cultured chondrocyte sheets. There is also a time lag needed for the culture phase, and the procedure is expensive. Autografting is considered essential for the establishment of allogeneic transplantation, and cell culture is necessary in developing methods for storing chondrocyte sheets. The use of vitrified–thawed chondrocyte sheets can address these problems. We have already developed cryopreservation by vitrification that enables the preservation of chondrocyte sheets, and the safety of the method has been demonstrated *in vitro* (Maehara *et al.*, 2013). This study examined the efficacy of using such cryostored chondrocyte sheets in treating full-thickness articular defects induced in the knees of rabbits.

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 2010), published by the National Institutes of Health, USA, and the Guidelines of Tokai University on Animal Use (Authorization No. 131031).

2.1. Temperature-responsive culture dishes

The temperature-responsive culture dishes (UpCell; provided by CellSeed, Tokyo, Japan) are coated with poly(N-isopropylacrylamide), which can change from being hydrophilic to being hydrophobic depending on the temperature, and were developed by Okano *et al.* (1993, 1995). This polymer facilitates cell adhesion and growth in normal culture conditions at 37°C. Reducing the culture temperature to <30°C causes the surface to hydrate and swell rapidly, prompting complete detachment of adherent cells without the need for typical treatment with proteolytic enzymes or trypsin. The culture dishes were sterilized using ethylene oxide gas (Sekiya *et al.*, 2006).

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

Six Japanese white rabbits (age, 16–18 weeks; weight ~ 3 kg) were used as the source of articular chondrocytes and synovial cells. Chondrocytes were harvested from the femur, and synovial cells were taken from inside the knee joints. After the cells had been isolated enzymatically, the chondrocytes were seeded onto temperature-responsive inserts, the synovial cells were placed into temperature-responsive culture dishes, and the cells were cocultured. Coculture using the inserts reduced the proliferation time compared with that required for culturing each type of cell independently (Ito *et al.*, 2012; Kokubo *et al.*, 2013; Takaku *et al.*, 2014). The harvested cartilaginous and synovial tissues were sliced finely with scissors and incubated on Petri dishes in Dulbecco"s modified Eagle"s medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) containing 0.5% (w/v) collagenase type 1 (Worthington, Lakewood, NJ, USA) at 37°C, 5% CO_2 in humidified air for 4 h while being stirred mechanically and the proteins degraded.

2.3. Cell culture using temperature-responsive culture dishes

The chondrocytes and synovial cells were passed through a cell strainer (BD Falcon Labware, Franklin Lakes, NJ, USA) with a pore size of 100 μm and the cells were retrieved by centrifugation at 518 g for 10 min. The synovial cells were maintained in a culture medium of DMEM/F12 supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics–antimycotics (Gibco); from day 4 onward, the culture was maintained by adding a further 50 μg/ml ascorbic acid (Wako Junyaku Kogyo, Osaka, Japan). The chondrocytes were incubated in a culture medium of DMEM/F12 supplemented with 20% FBS and 1% antibiotics–antimycotics; from day 7 onwards, the culture was maintained by adding a further 50 μg/ml ascorbic acid. All culturing was performed at 37°C, in 5% CO_2 and 95% humidified air. The chondrocytes were seeded on temperature-responsive inserts (4.2 cm^2; CellSeed) and the synovial cells were seeded in temperature-responsive culture dishes (9.6 cm^2; CellSeed) and cocultured for 14 days. The chondrocytes were seeded at a density of 50 000 cells/cm^2 and the synovial cells were seeded at 10 000 cells/cm^2 (Hamahashi *et al.*, 2015; Ito *et al.*, 2012).

2.4. Chondrocyte sheet retrieval

After the cells had been cultured for 2 weeks, they reached confluency and the temperature-responsive inserts were taken out of the incubator and left for 30 min at 25°C. After the culture medium had been removed, polyvinylidene fluoride (PVDF) membranes were used to retrieve the chondrocyte sheets, as described (Yamato *et al.*, 2001). Briefly, the chondrocyte sheet was covered with a PVDF membrane, and then both were harvested carefully as one unit. This facilitated good retrieval of the cultured chondrocyte sheets. Next, the PVDF membrane covering the chondrocyte sheet was harvested by rolling the membrane up at the edge of the culture dish, and then overlaying it on top of another chondrocyte sheet. This was performed three times to fabricate triple-layered chondrocyte sheets. Because the multilayered sheets floated in culture medium, cell strainers (BD Falcon Labware) were placed on top of them to prevent this. The layered chondrocyte sheets were further cultured for 1 week in temperature-responsive culture dishes.

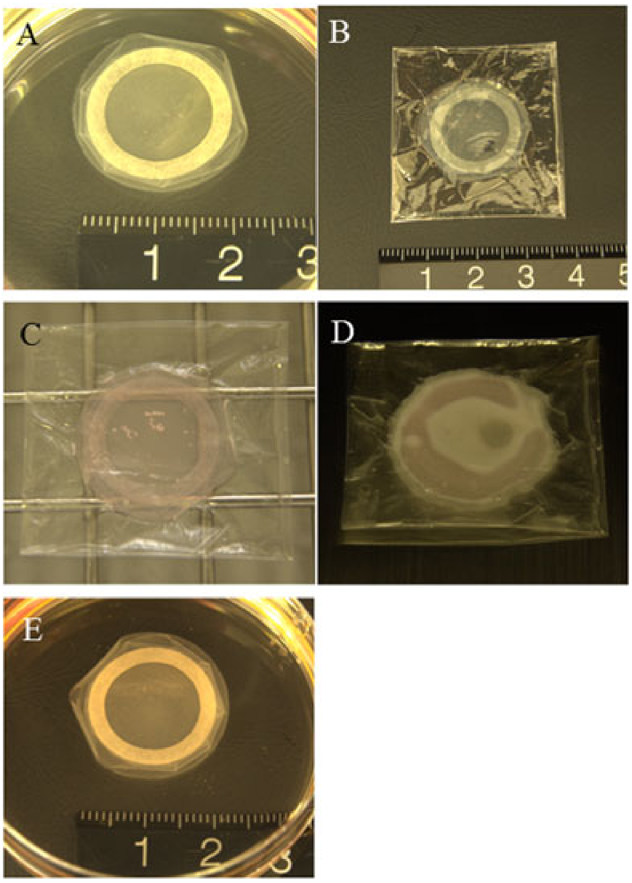

Figure 1. Vitrification and rewarming methods for triple-layered chondrocyte sheets. In A, B, and E, each ruler shows the scale in cm. (A) A layered chondrocyte sheet in vitrification solution treatment before vitrification. (B) This was covered with a wrapping film. (C) The wrapped sheet was vitrified by exposure to liquid nitrogen. (D) The vitrified sheet was placed onto a heating plate to rewarm. The white appearance is caused by frost around the wrapping film. (E) After thawing, the vitrified sheet was soaked in washing solution to dilute and remove the cryoprotectants [Colour figure can be viewed at wileyonlinelibrary.com]

2.5. Vitrification solutions

Cryopreservation by vitrification (Figure 1) of the layered chondrocyte sheets was performed as reported (Maehara *et al.*, 2013). Hepes (20 mM)-buffered tissue culture medium-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 20% calf serum (12133C; SAFC Biosciences, Lenexa, KS, USA) was used as the basal solution. Dimethyl sulfoxide (DMSO) and ethylene glycol (EG) were used as permeable cryoprotectants (CPAs). Sucrose and carboxylated poly-L-lysine were used as nonpermeable CPAs. An equilibration solution (ES) consisting of 10% (*v*/v) DMSO and 10% (*v*/v) EG in the basal solution and a vitrification solution (VS) containing 20% (*v*/v) DMSO, 20% (*v*/v) EG, 0.5 M sucrose, and 10% (*w*/*v*) and carboxylated poly-L-lysine were prepared. A rewarming solution (RS) and a dilution solution containing 1 or 0.5 M sucrose, respectively, were prepared and the basal solution was used as the washing solution (WS). The VS was used on crushed ice and RS was prewarmed to 38°C and used to thaw the vitrified chondrocyte sheet. All other solutions were used at room temperature (24–27°C).

2.6. Vitrification and rewarming procedures

First, vitrification and rewarming of the chondrocyte sheets were carried out as described (Maehara *et al.*, 2013). Briefly, a triple-layered chondrocyte sheet was peeled from the UpCell surface using a cell shifter and forceps, and immersed in 5 ml of ES in a 60 mm dish (Iwaki 3010–060; AGC Techno Glass, Shizuoka, Japan) for 5 min for pre-equilibration. Then, for dehydration and equilibration with the permeable CPAs, the chondrocyte sheet was transferred to the same solution in a fresh dish for 20 min. After the first equilibration period, the chondrocyte sheet was transferred to VS using forceps for 5 min (VS pretreatment), and then transferred to fresh VS in another dish for 15 min for further dehydration and equilibration with the permeable CPAs.

A chondrocyte sheet pretreated with ES and VS as described above was placed onto a 5 × 10 cm rectangular piece of polyvinylidene chloride kitchen wrap (Kureha Corporation, Tokyo, Japan) using forceps. Then, the wrapping film was folded to enclose the chondrocyte sheet. The wrapped sheet was held 1 cm above the surface of liquid nitrogen (LN_2) and vitrified by exposure to the vapor for 20 min. The vitrified sheet was gently transferred into a cell storage box (SPL-80025G, 1.5 ml; SPL Life Science, Seoul, Korea) and then into the LN_2 tank. Here, the vitrified sheet was cryopreserved through storage in the evaporated vapor layer of LN_2 (–150°C) for 4 weeks.

To thaw the vitrified sheet, the chondrocyte sheet envelope was placed directly onto an electric heating plate (HP-4530; ASONE Corporation, Osaka, Japan) at 38°C for 90 s. When the sheet had completely devitrified, the wrapping film was opened slowly and the sheet transferred into RS using forceps. The recovered sheet was checked for cracks, and the CPAs were diluted and removed in a stepwise manner. Briefly, the chondrocyte sheet was held in RS for 1 min and then transferred using forceps into 5 ml of dilution solution for 3 min. Then, the chondrocyte sheet was transferred twice into 5 ml of WS. The chondrocyte sheet was shaken gently several times in each solution and the sheets were kept in the first and second batches of WS for 5 min each.

2.7. Transplantation of layered chondrocyte sheets

Eighteen Japanese white rabbits (female, age 16–18 weeks, weighing ~3 kg; six in each of three groups) were used in this study. The rabbits were anesthetized using isoflurane, N_2O, and O_2. On the right side, a medial parapatellar incision was made; the patella was moved 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, confirming that an osteochondral defect had been produced. To obtain further coverage, layered chondrocyte sheets were grafted onto the defect. The rabbits underwent the following three treatments: group A received osteochondral defects only (control group); group B received osteochondral defects plus conventional fresh chondrocyte sheets; and group C received osteochondral defects plus vitrified–thawed chondrocyte sheets. After the surgery, all of the rabbits were returned to their cages without splinting or immobilization.

2.8. Pain evaluation

One week after transplantation, an incapacitance tester (Linton Instrumentation, Norfolk, UK) was used to determine any trends in the ratio of weight distribution between the undamaged and damaged limbs, and these trends served as the gauge for evaluating pain. This device facilitates automatic and reproducible pain evaluation by measuring the distribution of load between the hind limbs: a dual-channel weight-averaging technique. It is used widely to investigate pain-ameliorating effects in animal models (Ito *et al*., 2012; Mihara *et al*., 2007). To acclimatize the animals to the incapacitance tester, each day for 7 days after they were delivered, they were all placed in the main container (holder) of the device and held still for 5 s. The measurements were conducted while the rabbits were immobile after they were transferred into the rabbit holder, and when they were immobile after being removed from and then returned to the holder. This process was carried out five times, and the weight distribution between the hind legs was calculated using the following formula: damaged limb weight distribution ratio (%) = [damaged limb load (g) / undamaged limb load (g) + damaged limb load (g)] × 100. This measurement was taken as the mean of five repeats. After surgery, the measurements were performed eight times at weeks 1, 2, 3, 4, 6, 8, 10 and 12.

2.9. Histological evaluation of cartilage repair

The rabbits were euthanized at 12 weeks by an intravenous pentobarbital overdose. The transplanted tissue was removed from the distal portions of the unilateral femurs. The tissue samples were fixed in 4% paraformaldehyde for 1 week. Then, they were decalcified for 2–3 weeks using distilled water (pH 7.4) containing 10% ethylene diamine tetra-acetic acid. The tissues were then embedded in paraffin wax and sectioned perpendicularly (8 mm sections) through the centre of the defect. Each section was stained with Safranin O for glycosaminoglycans. Immunostaining was performed as described (Ito *et al*., 2012; Nagai *et al*., 2008b). Briefly, dewaxing was performed using standard procedures before immunostaining the sections. They were treated with 0.005% proteinase (type XXIV; Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. After washing the sections in phosphate-buffered saline (PBS), they were treated with 0.3% hydrogen peroxide/methanol solution at room temperature for 15–20 min to activate endogenous peroxidase. The sections were then reacted for 30 min in normal goat serum diluted 1:20 in PBS. Mouse primary monoclonal antibodies reacting with human types I and II collagen (Daiichi Fine Chemical Co. Ltd., Toyama, Japan) were then diluted 1:200 with PBS plus 1% bovine serum albumin (BSA; Sigma–Aldrich). 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, which were diluted with 1% BSA/PBS at a dilution of 1:100. After that, the sections were treated for 1 h with horseradish peroxidase and stained with streptavidin–horseradish peroxidase. They were then 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 haematoxylin to enhance cell visibility.

In the histological evaluation, scoring was carried out blind by three examiners using a modified form of

Safranin O staining outcome as reported (O'Driscoll *et al.*, 1998) and the International Cartilage Repair Society (ICRS) grading system (Brehm *et al.*, 2006; Mainil-Varlet *et al.*, 2003).

2.10. Statistical analysis

Analysis of variance (ANOVA) was used to analyse the rate of loading 12 weeks after surgery and the histological appraisal scores. The Tukey–Kramer method was used for *post hoc* testing. The results are expressed as the mean ± standard error (SE), and $p < 0.05$ was assumed to be statistically significant.

3. Results

3.1. Damaged limb weight distribution ratios

Figure 2A shows the damaged limb weight distribution ratio (mean ± SE) in weeks 1, 2, 3, 4, 6, 8, 10 and 12 after graft surgery. Group A showed poor improvement: 36.3 ± 2.3% to 41.3 ± 0.4% in week 12 compared with the ratio measured immediately after surgery. By contrast, groups B and C showed improvements in the ratios as follows: group B 35.6 ± 1.1% to 49.3 ± 0.1%; group C 37.7 ± 1.2% to 48.9 ± 0.1%. Figure 2B shows the damaged limb weight distribution ratios in week 12 after surgery. Groups A and B showed significant differences using the Tukey–Kramer test, as did group A vs. group C. No significant differences were observed between groups B and C.

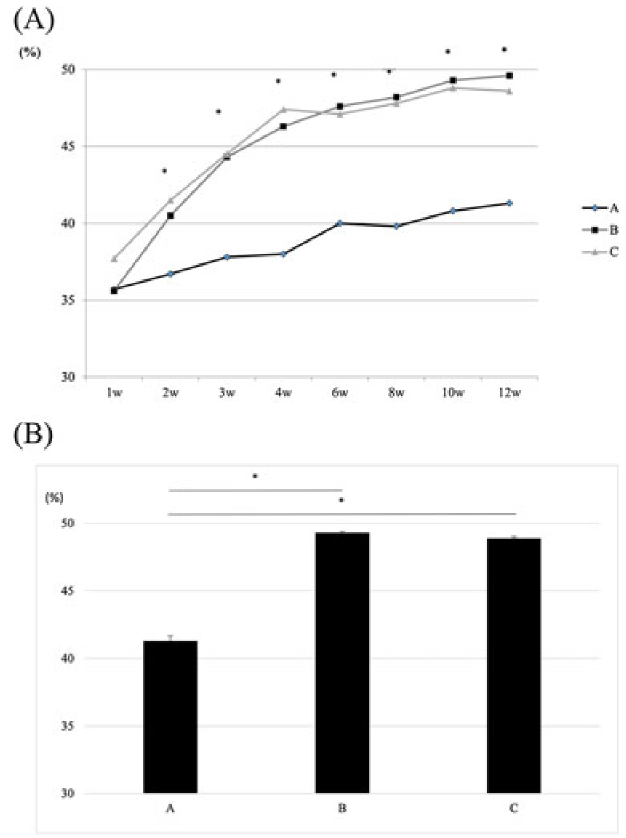

Figure 2. Pain alleviation effect. (A) The results for the damaged limb weight distribution ratio after surgery. Group A had an osteochondral defect only (control); group B received chondrocyte sheets; group C received vitrified chondrocyte sheets. Damaged limb weight distribution ratio (%) = [damaged limb load (g) / undamaged limb load (g) + damaged limb load (g)] × 100. From 2 weeks after surgery, significant differences were observed between groups A and B, and between groups A and C (*$p < 0.05$). In group A at 1 week after surgery, the damaged limb weight distribution ratio was 36.3 ± 2.3%, and by 12 weeks this had barely improved to 41.3 ± 0.4%. Group B improved from 35.6 ± 1.1% to 49.3 ± 0.1%, and group C improved from 37.7 ± 1.2% to 48.9 ± 0.1%. (B) Damaged limb weight distribution ratio (%) at 12 weeks after surgery: group A was 41.3 ± 0.4%, group B was 49.3 ± 0.1%, and group C was 48.9 ± 0.1%. Groups B and C had significantly higher ratios than group A (*$p < 0.05$). No significant differences were observed between groups B and C [Colour figure can be viewed at wileyonlinelibrary.com]

3.2. Gross findings in the repaired cartilage

All defects were filled with cartilaginous tissue, but repair in group A was not sufficient. The surface layers in groups B and C had been replaced with smooth cartilaginous tissue with a colour resembling that of intact cartilage, but the surface layer in group A had been replaced with poor fibrous tissue, while the subchondral bone appeared through the defect in some parts.

3.3. Histology of repaired tissues

The operations were uneventful and all of the rabbits were returned to their cages and allowed to act freely. We did not find any signs of infection. Twelve weeks after surgery, six knees from each group were evaluated after the animals had been euthanized. Figure 3 shows a histological image of repaired tissue immunostained and counterstained with Safranin O. We evaluated the tissues using the ICRS histological grading system (Brehm *et al.*, 2006; Mainil-Varlet *et al.*, 2003; O'Driscoll *et al.*, 1998), which is a modification of that reported by O'Driscoll *et al.* (1998). This system evaluates repaired tissue based on 11 items: tissue morphology, matrix staining, structural integrity, cluster formation, tidemark opening, bone formation, histological appraisal of surface architecture, histological appraisal of the degree of defect filling, lateral integration of defect-filling tissue, basal integration of defect-filling tissue, and histological signs of inflammation. The total score range was 11–45 (Table 1).

Figure 4 and Table 2 show the ICRS grading system results 12 weeks after surgery. The results were: group A, 15.2 ± 0.2; group B, 40.0 ± 0.6; and group C, 39.0 ± 0.4. Significant differences were observed between groups A and B, and between groups A and C ($p < 0.05$). No significant differences were observed between groups B and C. Groups B and C exhibited significantly higher scores than group A except for the histological signs of inflammation factor. Values are the mean ± SE. The total score range is from 11 (no repair) to 45 (normal articular cartilage). Groups B and C exhibited significantly higher total scores than did group A ($p < 0.05$).

3.4. Immunohistochemistry

Figure 3 shows repair tissue immunostained 12 weeks after surgery. Type II collagen expression was observed in

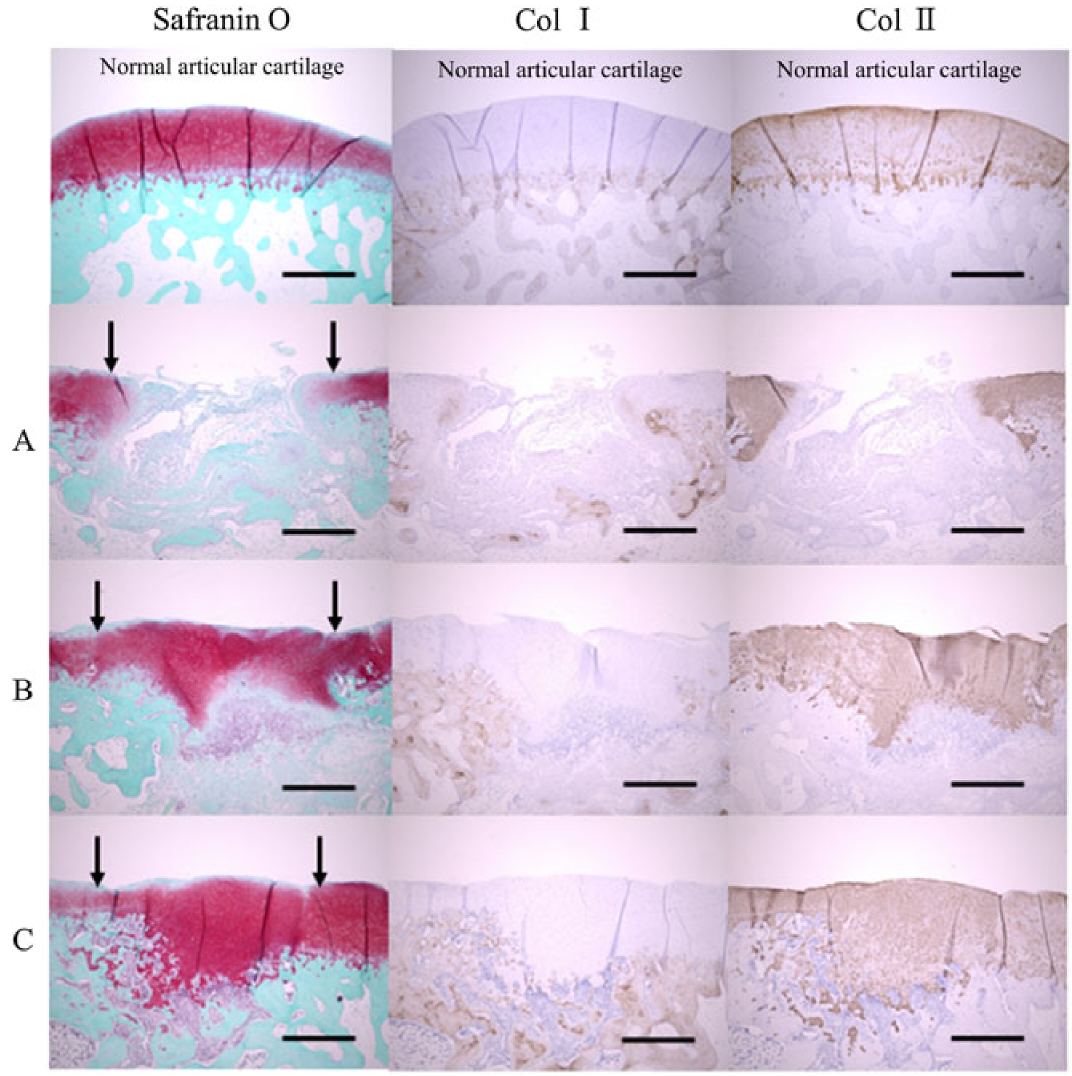

Figure 3. Histology of repaired tissues at 12 weeks after surgery. In group A, the defects had not been filled with repair tissue. In group B, the formation of convex repair tissue was achieved, and structural consistency, defect filling rates, and condition of the superficial layer of the defect were good. In group C, the condition of the implanted tissue, tissue filling rates, and subchondral bone repair were good; the transplanted cartilaginous layer exhibited a columnar organization and had been repaired with hyaline cartilage that appeared almost intact. In groups B and C, we observed type II collagen immunostaining (brown) in tissue costained with Safranin-O. Type II collagen was expressed uniformly in the surrounding cells (images in the right columns), which made the cartilaginous repair tissue borders clearer. In contrast, in group A, no type II collagen expression was observed in the portion of the defect that had been replaced with fibrous tissue (bars =1000 μm) [Colour figure can be viewed at wileyonlinelibrary.com]

the grafted tissue and normal pericellular matrix of groups B and C. No type II collagen expression was observed in the defect in group A; instead, type I collagen expression was observed in the superficial portion of fibrocartilage and the superficial layer of subchondral bone.

4. Discussion

There has been widespread clinical use of cell grafting for repairing articular cartilage (Ando *et al*., 2007; Crawford *et al*., 2009; Koga *et al*., 2008; Moseley *et al*., 2010; Ochi *et al*., 2002; Zaslav *et al*., 2009). The indications for these treatments are small traumatic lesions, but it is necessary to investigate therapies for cases of OA, which involves variations in the size and depth of damage. Here we sought to repair articular cartilage, aiming for repair with complete hyaline cartilage rather than with fibrous cartilage.

The current authors have reported that layered chondrocyte sheets can repair defects with hyaline cartilage in rats (Takaku *et al*., 2014), rabbits (Ito *et al*., 2012) and minipigs (Ebihara *et al*., 2012). Other reports studied the mechanism of action and examined various humoral factors released or inhibited by chondrocyte sheets (Hamahashi *et al*., 2015), and the optimal conditions for human chondrocyte culture (Kokubo *et al*., 2013). Here, it is confirmed that pain alleviation and better tissue repair could be achieved using vitrified–thawed chondrocyte sheets, and the results are similar to those using conventional fresh chondrocyte sheets.

The authors have been carrying out clinical studies of joint repair using autologous chondrocyte sheets and excellent results have been achieved, but there are some problems. These include the need for two operations to harvest the chondrocytes and synovial cells and then to transplant the chondrocyte sheets, and about 3–4 weeks to culture the cells. In addition, implementation of

Table 1. ICRS histological grading system

Item	Description
Ti: Tissue morphology	1: Exclusively not cartilage 2: Mostly not cartilage 3: Mostly fibrocartilage 4: Mostly hyaline cartilage
Matx: Matrix staining	1: None 2: Slight 3: Moderate 4: Strong
Stru: Structural integrity	1: Severe disintegration 2: Cysts or disruptions 3: No organization of chondrocytes 4: Beginning of columnar organization of chondrocytes 5: Normal, similar to healthy mature cartilage
Clus: Chondrocyte clustering in implant	1: 25–100% of the cells clustered 2: < 25% of the cells clustered 3: No clusters
Tide: Intactness of the calcified cartilage layer, formation of tidemark	1: < 25% of the calcified cartilage layer intact 2: 25–49% of the calcified cartilage layer intact 3: 50–75% of the calcified cartilage layer intact 4: 76–90% of the calcified cartilage layer intact 5: Completely intact calcified cartilage layer
Bform: Subchondral bone formation	1: No formation 2: Slight 3: Strong
SurfH: Histological appraisal of surface architecture	1: Severe fibrillation or disruption 2: Moderate fibrillation or irregularity 3: Slight fibrillation or irregularity 4: Normal
FilH: Histological appraisal defect filling	1: < 25% 2: 26–50% 3: 51–75% 4: 76–90% 5: 91–110%
Latl: Lateral integration of implanted material	1: Not bonded 2: Bonded at one end/partially both ends 3: Bonded at both sides
Basl: Basal integration of implanted material	1: < 50% 2: 50–70% 3: 70–90% 4: 91–100%
InfH: Inflammation	1: No inflammation 3: Slight inflammation 5: Strong inflammation
Maximum total	45 points

This system evaluates repaired tissue based on 11 items: tissue morphology (Ti); matrix staining (Matx); structural integrity (Stru); cluster formation (Clus); tidemark opening (Tide); bone formation (Bform); histological appraisal of surface architecture (SurfH); histological appraisal of the degree of defect filling (FilH); lateral integration of defect-filling tissue (Latl); basal integration of defect-filling tissue (Basl); and histological signs of inflammation (InfH). The total score range is 11–45.

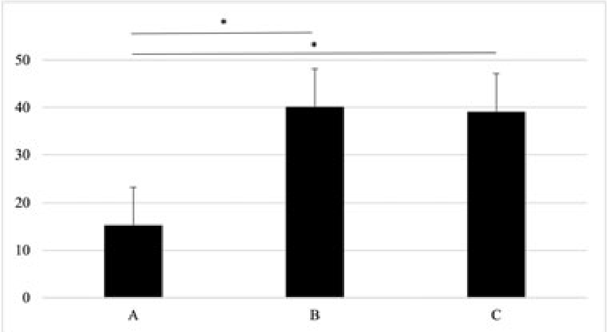

Figure 4. The results of the ICRS grading system at 12 weeks. Group A, 15.2 ± 0.2; group B, 40.0 ± 0.6; group C, 39.0 ± 0.4. Groups B and C exhibited significantly higher scores than group A (*$p < 0.05$). No significant differences were observed between groups B and C

multiple operations is difficult because the amount of intact articular cartilage is limited, the proliferative ability of human chondrocytes varies between individuals and declines with age, and such procedures are expensive.

Table 2. ICRS grades 12 weeks after surgery in the three groups of rabbits

Item	A	B	C
Tissue morphology (Ti)	1.50 ± 0.2	3.84 ± 0.2	4.00 ± 0.0
Matrix staining (Matx)	1.67 ± 0.0	4.00 ± 0.0	3.84 ± 0.2
Structural integrity (Stru)	1.17 ± 0.1	4.33 ± 0.3	4.16 ± 0.2
Cluster formation (Clus)	1.17 ± 0.1	3.33 ± 0.3	3.16 ± 0.2
Tidemark opening (Tide)	1.33 ± 0.0	4.50 ± 0.5	3.84 ± 0.2
Bone formation (Bform)	1.00 ± 0.0	2.67 ± 0.3	3.00 ± 0.0
Histological appraisal of surface architecture (SurfH)	1.17 ± 0.1	2.84 ± 0.2	3.00 ± 0.0
Histological appraisal of the degree of defect filling (FilH)	1.00 ± 0.0	4.84 ± 0.2	4.50 ± 0.2
Lateral integration of defect-filling tissue (Latl)	1.00 ± 0.0	3.17 ± 0.2	3.16 ± 0.2
Basal integration of defect-filling tissue (Basl)	1.17 ± 0.1	3.84 ± 0.2	3.50 ± 0.5
Histological signs of inflammation (InfH)	3.00 ± 0.0	3.00 ± 0.0	3.00 ± 0.0
Total scores (Hgtot)	15.2 ± 0.2	40.4 ± 0.6	39.1 ± 0.4

Group A, osteochondral defect only; group B, chondrocyte sheets; group C, vitrified chondrocyte sheets.

To address these problems, there is a need to establish allogeneic chondrocyte sheet transplantation and preservation techniques for general application. Articular cartilage is one of few tissues not requiring immunosuppression for such transplantation (Fragonas *et al*., 2000; Kawamura *et al*., 1998), and it has already been confirmed that the use of chondrocyte sheets does not cause immune rejection in the recipient (data not shown). The method of vitrifying chondrocyte sheets used here did not cause damage or loss of major biological components in a previous study (Maehara *et al*., 2013). In the conventional slow-freezing method, cultured cell sheets are frozen in the presence of relatively low concentrations of CPAs, and the formation of extracellular and intracellular ice crystals is inevitable during freezing. This destroys the sheet structure and decreases cell viability (Kito *et al*., 2005). In contrast, with vitrification, a solution containing a high concentration of CPAs is cooled rapidly to achieve a transition from a liquid phase to an amorphous glassy solid phase without ice crystal formation (Rall and Fahy, 1985). In this way, chondrocyte sheets can be sealed in a state that maintains their macro- and microstructures and sustains high cell viability after thawing. Moreover, vitrification is much quicker than slow freezing and does not need an expensive freezer with a calibrated cooling control device. Further, it is capable of managing significant amounts of tissue for allogeneic transplantation, so it is safe and economical because the safety evaluation that must be performed each time during autologous transplantation can be omitted.

This study has confirmed that this vitrification method might enable long-term cryostorage of the chondrocyte sheets without affecting their therapeutic benefits. Thus, it is anticipated that this approach will be efficacious for allogeneic chondrocyte sheet transplantation. There are plans to investigate protocols and safety issues further.

5. Conclusions

In this rabbit model of an osteochondral defect, significantly better pain-alleviating effects and tissue repair were achieved by using vitrified–thawed chondrocyte sheet transplantation compared with no treatment (an osteochondral defect alone). No significant differences were observed between the transplantation of conventional fresh chondrocyte sheets and vitrified chondrocyte sheets.

Author disclosure statement

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Conflicts of interest

The authors declare no conflicts of interest.

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