Vitreous preservation of articular cartilage from cryoinjury in rabbits

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【Abstract】

Frozen osteoarticular grafts treated with liquid nitrogen are utilized for joint reconstruction after tumor resection, but the joints may subsequently develop osteoarthritic changes. To preserve articular cartilage from cryoinjury, we modified a vitrification method utilized for embryo cryopreservation and demonstrated *in vitro* that our vitrification protocol was effective for protecting cartilage from cryoinjury. In this study, we investigated *in vivo* whether this vitrification method could protect against

osteoarthritic changes in articular cartilage. Osteochondral plugs were obtained from the distal femur of rabbits. These grafts were divided into 3 groups: Fresh group (F-group), non-vitrification group (N-group), and vitrification group (V-group). After treatment, the plugs were re-implanted as autografts. Histological findings, chondrocyte viability, and ultrastructural examinations were examined 6, 12, and 24 weeks after implantation. Histological findings of chondrocytes for the V- group showed no significant difference from those of the F-group at any time point except at 24 weeks postimplantation at the non-weight bearing site ($p<0.05$).Viability of chondrocyte showed no significant difference from those of the F-group except at 12 weeks postimplantation at the bearing site ($p<0.05$). In contrast, viable cells disappeared from the N-group and histology and viability significantly differed between the N-group and the V-group. Transmission electron microscopy demonstrated preservation of chondrocyte structure in the V-group and the F-group, but chondrocytes of the N-group were abnormally electron dense . Our vitrification method was effective in protecting chondrocytes from cryoinjury that might lead to cartilage degeneration. Reconstructing joints with osteoarticular grafts containing living cartilage may help to avert osteoarthritic changes. Our vitrification method could prove useful for reconstruction with frozen tumor-containing autografts and for long-term storage of living cartilage for allografts.

【Introduction】

Improvements in survival rates associated with the advent of multidisciplinary treatments for sarcomas have necessitated the development of functional and durable methods of joint reconstruction after tumor resection. Massive prostheses and allografts are the two standard approaches to managing bone defects after tumor excision. Reconstructions with massive prostheses provide immediate postoperative stability and an early return to daily activities, but recipients are at risk for late complications such as aseptic loosening, mechanical failure and, especially in young patients, bone loss[2,5,26]. Reconstructions with allografts are being performed in some countries, but the procedure is not acceptable in certain Asian countries, particularly in Japan, because of socio-religious reasons. Therefore, in order to offer patients another alternative, various devitalizing methods, including irradiation[32], autoclaving[12,31], and pasteurization[16] have been investigated to enable re-use of resected tumor-containing bone in joint reconstruction.

We have developed a new reconstruction method using frozen autografts in which the resected tumor-containing bone is immersed into liquid nitrogen (LN_2) for 20 min to kill tumor cells, after which the tissue is re-grafted at the same location[33]. An *in vitro* study verified that our liquid nitrogen treatment method totally devitalized sarcoma

tissues such as osteosarcoma[36]. We have performed reconstructions with frozen autografts since 1999, and no recurrences of sarcomas from the grafted bone have occurred. The advantages of our freezing method include the following: simplicity, osteoinduction, osteoconduction, a perfect fit, sufficient biomechanical strength, and induced cryoimmunological anti-tumor effects[20,36]. Unfortunately, joints that have been reconstructed with frozen autografts may later develop osteoarthritic changes, a complication that also can occur following other biological reconstructions[16,37].

We thus initiated research to clarify the cause of joint degeneration in reconstructions using frozen autografts and to identify methods for preventing this complication. Our investigations found that frozen grafts were incorporated into the host bone and that excellent remodeling occurred in frozen bone. On the other hand, cartilage degeneration progressed in the frozen bone grafts, although the structures of the other joint components were well-preserved[7]. Previous study have reported cartilage degeneraton caused by cryoinjury, which caused ice crystal formation [34].

To preserve articular cartilage from cryoinjury, we sought to develop a method to protect cartilage selectively from cryoinjury, which is one of the causes of the subsequent osteoarthritic changes. Some investigators have examined cryoprotectants and cryopreservation protocols in an effort to enable preservation of viable cells during

freezing-thawing[1,24] . However, preserving the viability of structured tissues and organs is difficult using conventional approaches because insufficient amounts of cryoprotectant are not able to penetrate cells deep in the matrix to prevent cryoinjury[8,21]. Recently, a vitrification method was developed in the field of assisted reproductive technology for cryopreservation of oocytes and embryos[13,22,29]. Vitrification, which is promoted by a high concentration of cryoprotectants during cooling, results in a high percentage of cells retaining viability after thawing. We have modified a vitrification method utilized oocyte cryopreservation and demonstrated *in vitro* that our vitrification protocol was effective for protecting cartilage from cryoinjury[7]. Recent investigations on vitrification of articular cartilage have demonstrated protection of chondrocytes from cryoinjury *in vitro* [6,28,35]. One study with vitrification *in vivo* demonstrated protection of cartilage from cryoinjury histologically[27]. However, demonstration of *in vivo* function of vitrified cartilage as an autograft has yet to be accomplished. *In vivo* function and recovery of viable chondrocytes will allow long term maintenance of the matrix [6].

The objective of this study was to examine whether our vitrification method can protect against osteoarthritic changes of articular cartilage and preserve function of chondrocytes after liquid nitrogen treatment *in vivo* in a rabbit model and to compare

the performance of fresh control and frozen cartilage.

【Materials and Methods】

Adult female Japanese White rabbits, weighing 2.5-3.5 kg, were used in this study. A total of 45 rabbits (5 rabbits in each of subgroups) were randomly allotted to 3 groups. Animals were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight; Sankyo, Tokyo, Japan) and intravenous injection of pentobarbital sodium (50 mg/kg body weight; Dainippon Pharmaceutical, Tokyo, Japan). The left hind limbs were used for surgical treatment while the right hind limbs were left intact.

Osteochondral plugs (2.7 mm diameter, 0.1 - 0.4 mm full-thickness articular cartilage on a 4 - 5 mm bone base) of anesthetized rabbits were obtained from the weight bearing site of the medial condyle of the femur and the non weight bearing site of the patellar groove of the femur using mosaicplasty (Smith & Nephew, Inc. Andover, MA) under sterile conditions. The plugs were transferred into phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) and stored until use in the experiments.

At the onset of the experiment, the plugs were divided randomly into 3 groups:

・Fresh group (F-group) - control group, using unfrozen, fresh cartilage plugs;

・Non-vitrification group (N-group) - direct immersion of plugs into LN_2 without any cryoprotectants for 20 min followed by warming to room temperature;

・Vitrification group (V-group) – pretreatment of plugs with cryoprotectants (see below) prior to immersion in LN_2 for 20 min followed by warming to room temperature.

The plugs in each group were reimplanted into the same site in the same animal. Histological findings, viability of chondrocytes, and ultrastructural findings in each group were examined at 6 weeks, 12 weeks and 24 weeks postimplantation.

Vitrification protocol

Osteochondral plugs were washed in fresh PBS. After washing, the plugs were equilibrated in PBS supplemented with 20% ethylene glycol (Wako Pure Chemical Industries, Osaka, Japan) and 0.3 M sucrose (Wako Pure Chemical Industries) for 2 min. Next, the plugs were exposed to the vitrification solution (PBS supplemented with 40% ethylene glycol and 0.6 M sucrose) for 2 min and then immediately immersed into LN_2 for 20 min. At the end of the 20 min period in LN2, the plugs were warmed for 5 min in PBS supplemented with 1 M sucrose and subsequently immersed in PBS for 5 more minutes to dilute the cryoprotectants.

Histological evaluations of osteoarthritic changes

To examine morphological changes in the cartilage, the constituent cells and extracellular matrix were examined by light microscopy. Specimens were prepared as described above and stained with hematoxylin and eosin or safranin-O. The Mankin score (Table1) [17] was used to evaluate the specimens, where higher numbers correspond with increasing amounts of osteoarthritic changes.

Viability assay of chondrocytes

To examine the viability of vitrified-warmed chondrocytes, the osteochondral plugs were washed 3 times in PBS and counterstained with 10 μg/mL bis-benzimide (Hoechst 33342; Sigma) and 10 μg/mL propidium iodide (Sigma) for 30 min. They were then washed in PBS and treated with anti-fading solution (Slow-Fade; Molecular Probes, Eugene, OR, USA). Thin sections (approximately 0.5 mm) were cut from the plugs and mounted on glass slides. Labeled chondrocytes were examined using a Keyence BZ 9000 microscope fitted with epifluorescence illumination. Viable cells emitted a blue fluorescence, whereas non-viable cells emitted a red fluorescence. The numbers of live and dead cells were counted. The percentage of live cells was used as a measure of viability. All sections were counted three times and the counts were averaged in order to calculate the percentage of live cells.

Ultrastructural examination

To examine the ultrastructure of chondrocytes in each group, the constituent cells and extracellular matrix were examined by transmission electron microscopy (TEM). Specimens were prepared as described above and fixed in 2% (v/v) glutaraldehyde in 2% paraformaldehyde-phosphate buffer at 4□, then post-fixed in 2% (v/v) osmium tetroxide in 0.1M phosphate buffer. These specimens were then dehydrated in ethanol, treated with n-butyl-glycidyl ether prior to embedding in Spurr's epoxy resin and cut into 1 mm cubes. One micrometer sections were then stained with 0.1% toluidine blue and examined by microscopy to identify areas of interest. Sections (80 nm) of suitable areas were cut and stained with uranyl acetate and lead citrate prior to examination by electron microscopy (H-7650, Japan). Approximately five to ten chondrocytes at various levels through these specimens were examined in detail.

Statistical analysis

Histopathology and histochemistry data were performed by one-way analysis of variance (ANOVA) followed by a post-hoc Fisher's protected least significant difference test (PLSD) using the Statview program (Abacus Concepts, Inc, Berkeley, CA, USA). Differences with a $P < 0.05$ were considered significant.

Viability data of the chondrocytes were subjected to arcsine transformation before analysis of variance (ANOVA). Transformed data were tested by ANOVA followed by

a post-hoc Fisher's protected least significant difference test (PLSD) using the Statview program (Abacus Concepts, Inc, Berkeley, CA, USA). Differences with a $P < 0.05$ were considered significant.

【Results】

Histological evaluations of osteoarthritic changes

Representative images of histological and histochemical findings for the three groups shown in Fig. 1. In the F group, there are some changes at all time points; the surfaces were irregular, and the staining intensity with the Safranin-O was reduced. The cell density was almost normal and in one case clonal proliferation was observed. Tidemark integrity was intact. In the V-group, there were changes at all time points; the cartilage surfaces were normal or irregular, and the Safranin-O staining intensity was reduced. The cell density was normal. Tidemark integrity was intact. In the N-group, specimens with severe osteoarthritis or absence of articular cartilage was observed at each time point; the surfaces were irregular, with clefts or completely disorganized, and the Safranin-O staining demonstrated severe reduction intensity or no staining noted. The explants were hypocellular. Tidemark integrity was disrupted, about 50% of crossed by blood vessels and about 50% of intact. Histological and histochemical findings were

similar between the F-group and the V-group at all time points. The Mankin scores of the three treatment groups are shown in Table 2. According to the Mankin scores, the total score of the V-group was significantly lower than that of the N-group at each time point, but there was no significant difference between the V-group and the F-group at any time point except at 24 weeks postimplantation at the non-weight bearing site.

Viability of chondrocytes

In the F-group, most cells stained blue and a few cells were stained red, indicating that the chondrocytes in the F-group were viable. Similarly in the V-group, a lot of cells were stained blue (viable) and a few cells were stained red (dead). In contrast, over 80% of grafts in the N-group had completely lost their cartilage and 0-30% of cells were stained blue. Quantitative viability assessment of the three treatment groups is shown in Table 3. The viability of the N-group was significantly lower than those of the V-group and the F-group at each time point. The mean viability of the V-group was more than 70% as well as the F-group. The difference between the F-group and V-group was not significant at any time point, except the viability of the V-group at the weight bearing site was significantly higher than that of the F-group at 12 weeks postimplantation.

Ultrastructural observations

The chondrocytes of the F-group and the V-group had sub-spherical nuclei, some cytoplasmic organelles and discrete plasma membrane with short cytoplasmic processes in each time point (Fig. 2A, B). On the other hend, few chondrocytes were detected in the N-group and they were abnormally electron dense (Fig. 2C). However, the matrix was similar in appearance to that seen in the other groups.

【Discussion】

Our investigation found that osteochondral grafts treated with liquid nitrogen after this vitrification method can preserve cartilage architecture similar to fresh osteochondral grafts upon histological evaluation at all time points. However, osteochondral grafts treated with liquid nitrogen alone developed osteoarthritic changes after re-implantation.

Studies with fresh autogeneous cartilage also demonstrated that the re-implanted cartilage appeared grossly normal and smooth[4,15] and the Safranin-O staining intensity of the implanted cartilage decreased[11],but these grafts showed some deterioration in the quantitative scale of cartilage degradation[18]. On the other hand, one study with cryopreserved autogeneous cartilage demonstrated that the re-implanted cartilage had fewer living chondrocytes and decreased safranin-O staining[4]. These

osteoarthritic changes might be caused by heat necrosis during the extraction of an osteochondral plug or due to the blunt impaction of the cartilage during the process of implantation[10]. Our vitrified cartilage showed some deterioration of cells, structure, and Safranin-O staining without cluster formation at all time points.

Ultrastructural observation is a traditional way to examine the effects of cryopreservation of articular cartilage. Preserved chondrocytes of allografts showed a prominent rough endoplasmic reticulum and sub-spherical nuclei upon ultrastructural observation [6,18,30]. On the other hand, degenerated chondrocytes had electron dense cytoplasm and irregular nuclei [6,14].

Although the extent and range of cryoinjury can be judged by ultrastructural and histological changes, it is difficult to make quantitative evaluations. One of the most important factors for achieving a successful clinical outcome after transplantation is maintaining the viability of cartilage. Therefore, viability assessment based on physical and biochemical properties of cells was performed by the Hoechst33342/Propidium iodide assay. In our previous work, we used this assessment to show that vitrified cartilage cell viability was >85% compared with fresh cartilage *in vitro*[7]. Other *in vitro* studies with vitrification reported 51-80% cell viability[6,28]. *In vivo* studies with fresh autografts and cryopreserved allografts reported that chondrocyte viability was

preserved for a short time[19,25]. The results of viability were comparable to *in vivo* data from a study using conventional cryopreservation protocols, which was evaluated by the metabolic status of articular cartilage[25].

Our findings suggest that this vitrification protocol was effective for protecting cartilage from cryoinjury and could preserve cartilage architecture and maintain function of chondrocytes. Vitrification, a cryopreservation method using a high concentration of cryoprotectant, can completely eliminate damage caused by ice crystal formation in the cytoplasm of cells during cryopreservation[9,27,35]. Past cryopreserved protocols consisted of slow freezing or a 2-step freezing. Slow freezing methods were developed to reduce ice crystal formation and to eliminate toxic and osmotic damage to cells through exposure to low concentrations of cryoprotectants while slowly decreasing the temperature[34]. However, it is difficult to completely eliminate intracellular ice formation by slow freezing alone as demonstrated in a study investigating cryopreservation of articular cartilage, where cryoinjury caused intracellular ice formation within chondrons, some of which occurred within chondrocytes[23]. In another vitrification study, damage to chondrocytes without cryoprotectant agent was far more pronounced than in those with vitrification[6]. Several studies have examined vitrification of cartilage under protocols using the VS55

formulation (2.2M propylene glycol, 3.1M formamide, and 3.1M Me_2SO) [6,27,28]. However, all of the vitrification protocols for cartilage to date are complicated and require more than 90 minutes for the stepwise addition and removal of cryoprotectants. We created a new protocol for cartilage preservation by modifying a vitrification method used for oocyte cryopreservation where we substituted ethylene glycol as the cryoprotectant and added it in two sequential 2-minute steps. Since our vitrification protocol is very simple and does not need specialized equipment and short time, our protocol is able to easily preserve viable cartilage. However, further investigations are necessary to determine whether this vitrification method can protect human articular cartilage from cryoinjury because it is thicker than rabbit cartilage. A more concentrated vitrification formulation was required for preservation of thicker cartilage. However, a large decrease in chondrocyte viability was observed when a vitrification protocol previously studied using rabbit cartilage was used for thicker porcine cartilage[3].

In reconstructions using frozen tumor-containing autografts, if the tumor is near the cartilage, we cannot use this vitrification method because it will also preserve tumor cells that might be present in the graft. However, if the cartilage is clearly isolated from the tumor and if we apply vitrification solution only to the cartilage, it may be possible to selectively protect the cartilage from cryoinjury.

In conclusion, our vitrification method appears to be effective for protecting rabbit cartilage from cryoinjury and preserving viable chondrocytes in osteochondral autografts. In addition, our vitrification method is superior to conventional cryopreservation as well as being very simple and easy to carry out. If human cartilage can be selectively protected from cryoinjury with vitrification, autologous frozen whole joint grafts could become an ideal approach for joint reconstruction after tumor resection. Moreover, vitrification might also be useful for long-term storage of living cartilage for allografts, tissue banking and cell banking.

【Acknowlegements】

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Figure legends

Fig.1. Histopathological and histochemistry findings from each experimental group at 24 weeks postimplantation. Full-thickness articular cartilage sections stained with Hematoxylin and eosin (HE) and Safranin-O. The bars represent 100 μm and 1000 μm, respectively. These sections were used to calculate Mankin scores as a measure of osteoarthritic changes. Implanted cartilage are between arrows. (A) (B) The Fresh group sections show Safranin-O staining and the Mankin score is 2, documenting that cartilage architecture was almost normal other than surface irregularity. (C) (D) The Vitrification group sections also show Safranin-O staining and the Mankin score is 1, documenting

that cartilage architecture was almost normal other than surface irregularity. (E) (F) The Non-vitrification group sections were devoid of chondrocytes and showed no Safranin-O staining of the matricies. The Mankin score was 8.

Fig.2. Ultrastructural findings from each experimental group at 24 weeks with the bar at the bottom representing 5μm.

(A) Chondrocytes in the F-group had sub-spherical nuclei and some cytoplasmic organelles and discrete plasma membrane with short cytoplasmic processes.

(B) Chondrocytes in the V-group also had sub-spherical nuclei and some cytoplasmic organelles.

(C) Chondrocytes in the N-group were abnormally electron dense.

Table.1 Mankin score

Ⅰ. Structure	Grade
a.Normal	0
b.Surface irregularities	1
c.Pannnus and surface irregularities	2
d.Clefts to transitional zone	3
e.Clefts to radial zone	4
f.Clefts to calcified zone	5
g.Complete disorganization	6
Ⅱ. Cells	
a.Nomal	0
b.Diffuse hypercellularity	1
c.Cloning	2
d.Hypocellularity	3
Ⅲ. Safranin-O staining	
a.Normal	0
b.Slight reduction	1
c.Moderate reduction	2
d.Severe reduction	3
e.No dye noted	4
Ⅳ. Tidemark integrity	
a.Intact	0
b.Crossed by blood vessels	1

Table 2. Mankin scores for each experimental group in weight-bearing sites (WB) and non-weight-bearing sites (NWB) at 6, 12, and 24 weeks (W) post-implantation

	6W		12W		24W	
	WB	NWB	WB	NWB	WB	NWB
F-group	4.2±1.6	3.2±1.1	2.8±0.4	3.8±1.9	3.8±4.6	2.6±1.5
V-group	3.0±0.7	1.8±0.4	3.0±1.0	2.2±1.1	2.0±0.0	1.0±0.7*
N-group	11.2±3.0**	9.2±2.6**	9.6±2.4**	8.2±1.3**	13.6±0.5**	13.6±0.5**

Data were expressed as the mean ± SD.
* Significantly different compared with F-group ($P < 0.05$).
** Significantly different compared with F-group and V-group ($P < 0.05$).

Table 3. Viability for each experimental group in weight-bearing sites (WB) and non-weight-bearing sites (NWB) at 6, 12, and 24 weeks (W) post-implantation

	6W		12W		24W	
	WB	NWB	WB	NWB	WB	NWB
F-group	88.5 (85.2, 91.8)	77.6 (72.9, 82.2)	71.4 (67.8, 74.8)	69.3 (63.7, 74.6)	88.5 (85.2, 91.8)	86.5 (82.1, 90.8)
V-group	86.5 (82.2, 90.8)	73.5 (67.7, 79.1)	81.4 (77.8, 85.0)*	70.9 (66.4, 75.2)	86.5 (82.2, 90.8)	87.5 (84.1, 90.9)
N-group	5.9 (−0.4, 12.2)**	5.2 (−0.3, 10.6)**	3.7 (−1.6, 9.0)**	0**	5.9 (−0.4, 12.2)**	0**

Data were expressed as the mean and 95% confidence limits.
* Significantly different compared with F-group ($P < 0.05$).
** Significantly different compared with F-group and V-group ($P < 0.05$).

Figure 1.

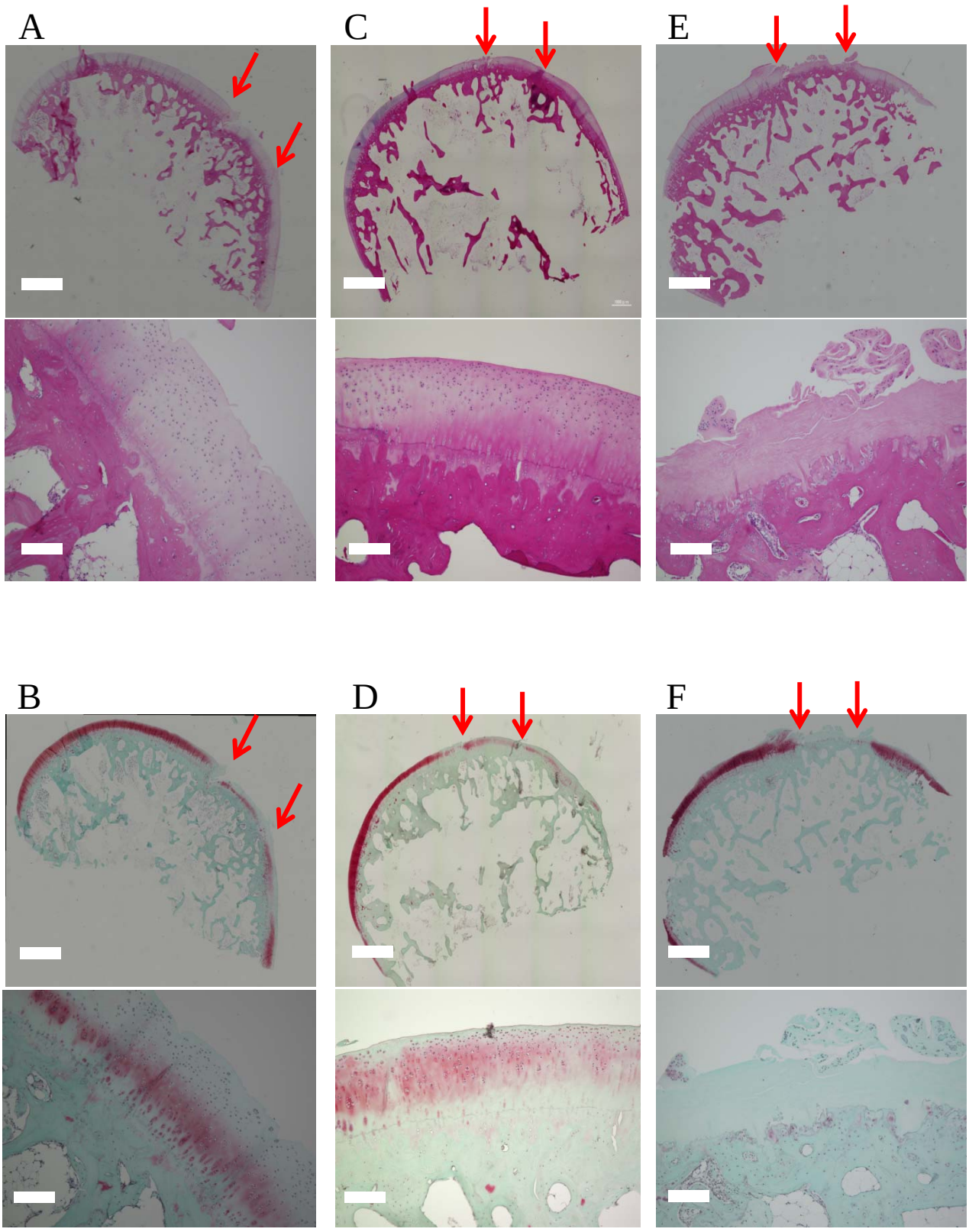
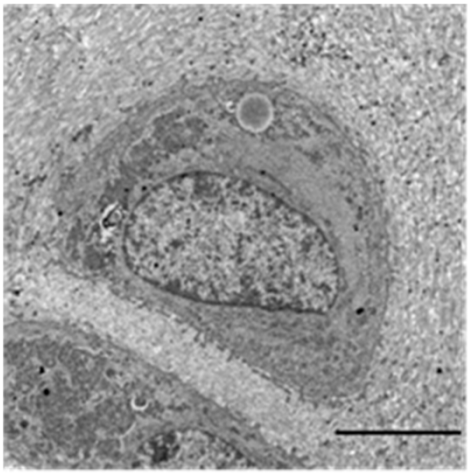
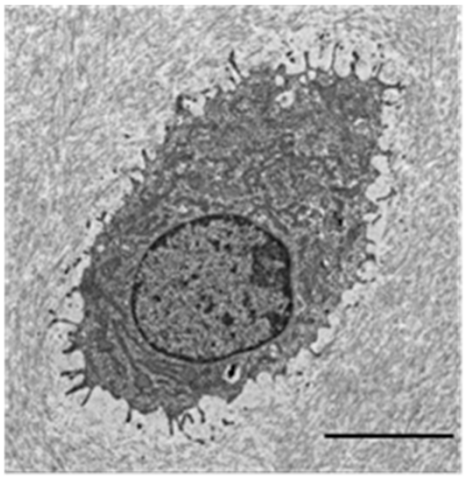


Figure 2.

A



B



C