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Abstract

Improving survival rates for sarcoma patients are necessitating more functional and durable methods of reconstruction after tumor resection. Frozen osteoarticular grafts are utilized for joint reconstruction, but the joint may develop osteoarthritic change. We used a frozen autologous whole-rabbit knee joint graft model to investigate the influence of freezing on joint components. Thirty rabbit knee joints that had been directly immersed into liquid nitrogen (L) or saline (C) without use of cryoprotectants were re-implanted. Histological observations were made after 4, 8, and 12 weeks. Both groups had bone healing. In group L, despite restoration of cellularity to the menisci and ligaments, no live chondrocytes were observed and cartilage deterioration progressed over time. It was concluded that cryoinjury of chondrocytes caused osteoarthritic change. Then we tested whether a vitrification method could protect cartilage from cryoinjury. Full-thickness articular cartilage of rabbit knee was immersed into liquid nitrogen with and without vitrification. Histology, ultrastructure, and chondrocyte viability were examined before and after 24 h of culture. Vitrified cartilage cell viability was > 85% compared with that of fresh cartilage. Transmission electron microscopy revealed preservation of original chondrocyte structure. Our vitrification method was effective for protecting chondrocytes from cryoinjury. Since reconstructing joints with osteoarticular grafts containing living cartilage avert osteoarthritic changes, vitrification method may be useful for storage of living cartilage for allografts or, in Asian countries, for reconstruction with frozen autografts containing tumors.

Keywords; Malignant bone and soft tissue tumor; Limb salvage; Reconstruction; Frozen autograft; Articular cartilage; Vitrification

Introduction

The survival rate of patients with sarcomas has been improved by multidisciplinary treatments and therefore functional and durable methods of joint reconstruction are necessary after tumor resection. Massive prostheses and allografts are standard approaches to manage bone defects after tumor excision. A massive prosthesis provides immediate postoperative stability and an early return to activities of daily living. Although the results of prosthetic reconstruction are favorable, patients have long-term risks for complications such as aseptic loosening, mechanical failure and, especially in young patients, bone loss [3,4,27]. Reconstruction with an allograft has been performed in some countries, but the procedure is not acceptable for some Asian countries, especially in Japan, because of socio-religious reasons. Therefore, various devitalizing methods have been developed for reusing resected tumor-containing bone for reconstruction including irradiation [36], autoclaving [14,35], and pasteurization [17]. 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 the tumor cells and then the tissue is re-grafted in the same location [37]. In our *in vitro* study, sarcoma tissue such as osteosarcoma was totally devitalized with our liquid nitrogen treatment method [40]. We have performed reconstruction with frozen autografts on 85 patients since 1999 and there are no recurrences of sarcomas from the grafted bone (the mean follow up 34 months; range 6-114). The advantages of this method are its simplicity, osteoinduction, osteoconduction, a perfect fit, sufficient biomechanical strength and anti-tumor effects by cryoimmunological function [20,40].

Frozen autografts can be utilized for joint reconstruction, but the joint may develop osteoarthritic changes, which can be observed in other biological reconstructions [17,41]. The objective of this research was to clarify the cause of joint deterioration and to prevent this complication. As a first step, we have developed an

autologous frozen whole-knee joint graft model in rabbits and examined the reintegration of grafts by radiological and histological methods to assess the effect of LN_2 on joint components.

As a next step, we considered a method to protect cartilage selectively from cryoinjury, which is one of the causes of osteoarthritic changes. Cryoinjury occurs as a result of the destructive effects of ice formation during freezing-thawing. Cryoprotectants and cryopreservation protocols enable the preservation of viable cells during freezing-thawing [2,23]. However, it is difficult to preserve the viability of structured tissues and organs by conventional approaches because adequate amounts of cryoprotectant can not penetrate the cells to prevent ice formation during freezing and thawing [13,21]. Recently, a vitrification method has been developed in the field of assisted reproductive technology for the cryopreservation of oocytes and embryos [16,22]. Vitrification, which is promoted by a high concentration of cryoprotectants during cooling, enables a high viability of cells after thawing. We modified a vitrification method utilized for embryo cryopreservation and examined whether this vitrification protocol is effective for the protection of cartilage from cryoinjury.

Materials and Methods

All experiments were performed following the guidelines for animal experiments established by the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government.

Experiment 1; Reconstruction with autologous frozen whole-knee joint in a rabbit model

Surgical methods

Adult female Japanese White rabbits, weighing 2.5–3.5 kg, were used in this study.

A total of 30 rabbits (5 rabbits in each of 6 subgroups) were randomly allotted to control (C) or LN_2-treated (L) groups. Animals were anesthetized with an intramuscular injection of ketamine hydrochloride (50 mg/kg body weight; Sankyo, Tokyo, Japan) and an intravenous injection of pentobarbital sodium (50 mg/kg body weight; Dainippon Pharmaceutical, Tokyo, Japan). The right hind limbs were used for surgical treatment while the left hind limbs were left intact.

The knee was exposed using a longitudinal medial parapatellar incision. The patellar tendon together with a fragment of the tibial tuberosity, which was detached with a thread wire saw, was reflected proximally. An osteotomy was then performed with a power saw at a level approximately 2.5 cm superior to the joint line in the femur. The knee joint containing intact ligaments and menisci was lifted free from the limb as continuity with the tibia was preserved. The leg was then rotated down. In the L group, the knee joint was immersed in LN_2 for 20 min and then thawed at room temperature. In the C group, the knee was immersed in saline for 20 min at room temperature. Fixation of the femur was achieved by osteosynthesis using 3 or 4 1.8-mm intramedullary Kirschner wires. The tibial tuberosity was replaced with a 1.5-mm cortical screw. A single prophylactic intramuscular injection of piperacillin sodium (5 mg/kg body weight; Toyama Chemical, Tokyo, Japan) was administered during surgery.

Postoperative management and evaluation

A bandage was placed over the incision site to allow wound healing. The animals were able to walk immediately after awakening. At each time point (4, 8, and 12 weeks after surgery), 5 rabbits from both the L and C groups were euthanized with an intravenous overdose of pentobarbital. We assessed reintegration of the grafts of the resected specimens *post mortem* by radiological, histological, and histochemical methods.

Radiographs were taken in 2 planes and evaluated for fusion, resorption, subchondral bone and fracture, fixation, subluxation, graft shortening, and narrowing of the joint space according to the criteria of the International Society of Limb Salvage (ISOLS) radiological implants evaluation system [5]. For histology and histochemistry, the resected specimens were fixed for 24 h in buffered formalin, decalcified with a 10% EDTA solution, and embedded in paraffin. Specimens were sectioned at a 5-μm thickness parallel to the bone axis and stained with hematoxylin, eosin, and safranin-O. The histomorphological findings of bony union, callus formation, and bone marrow were scored according to the system devised by Heiple *et al.* [9]. Histological and histochemical results were scored using the system of Mankin *et al.* [18] for the structure of cartilage, cellularity, safranin-O staining, and the integrity of the tidemark.

Experiment 2; Vitrification of articular cartilage discs for cryopreservation

Preparation of articular cartilage tissue

Osteochondral discs (3.0 mm diameter, 0.2-0.5 mm full-thickness articular cartilage on a 0.5-1 mm bone base) of adult female Japanese White rabbits were dissected from the femur using a scalpel under sterile conditions following euthanasia. The discs were transferred into phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA, USA) containing 0.3% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) and kept until used for the experiments.

Experimental groups

The discs were then divided randomly into 3 groups: Fresh group (F-group), some fresh cartilage discs were used as controls; Non-vitrification group (N-group), some discs were directly immersed into LN_2 without any cryoprotectants, kept in LN_2 for 20 min and then warmed to room temperature; Vitrification group (V-group), some discs

were vitrified, kept in LN_2 for 20 min and warmed according to the protocol described below. Transfer of discs into medium was performed using sterile forceps. The discs, with and without cryopreservation treatment, were cultured in PBS supplemented with 0.3% BSA and 50 μg/ml gentamicin (PBS-BSA) for 24 h at 38.5 °C under 5% CO_2 in air. The histology and histochemistry, ultrastructure, and chondrocyte viability of the discs in each group were examined before (groups F-1, N-1, and V-1) and after 24-h of culture (groups F-2, N-2, and V-2).

Vitrification protocol

Osteochondral discs were washed in fresh PBS-BSA. After washing, the discs were equilibrated in PBS-BSA supplemented with 20% ethylene glycol (Wako Pure Chemical Industries, Osaka, Japan) and 0.3 M sucrose (Wako Pure Chemical Industries) for 2 min. The discs were then exposed to a vitrification solution (PBS-BSA supplemented with 40% ethylene glycol and 0.6 M sucrose) for 2 min. After exposure to the vitrification solution, they were immediately immersed into LN_2 for 20 min. After vitrification, the discs were warmed for 5 min in PBS-BSA supplemented with 1 M sucrose and subsequently kept in PBS-BSA for 5 min to dilute the cryoprotectants. After dilution, the discs were transferred into fresh PBS-BSA and then cultured in the same medium for 24 h at 38.5 °C under 5% CO_2 in air.

Histopathology, histochemistry, and ultrastructure

To examine the morphological changes of the cartilage, the constituent cells and extracellular matrix were observed by light microscopy. Specimens were prepared as described above and stained with hematoxylin and eosin and safranin-O. We investigated the ultrastructural changes of cartilage after freezing with transmission electron microscopy. Cartilage slices were fixed in 4% glutaraldehyde in 0.1 M

phosphate buffer at 4°C and 2% osmium tetroxide in 0.1M phosphate buffer. Samples were then dehydrated in ethanol and treated with propylene oxide prior to embedding in Spurr's epoxy resin. Thin sections (80 nm) of suitable areas were cut and stained with uranyl acetate and lead citrate prior to examination with the electron microscope. We observed the chondrocytes and extracellular matrix at various levels in each slice in detail.

Viability assay of chondrocytes

To examine the viability of vitrified-warmed chondrocytes, the discs (4 discs per examination) before and after 24-h culture in each group were washed 3 times in PBS-BSA 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-BSA and treated with an anti-fading solution (Slow-Fade; Molecular Probes, Eugene, OR, USA). Thin sections (approximately 0.5 mm) were then cut from the discs and mounted on glass slides. Labeled chondrocytes were examined using a Nikon Diaphot microscope fitted with epifluorescence illumination. Live cells were distinguished by blue fluorescence and dead cells were identified by red fluorescence. The number of live and dead cells was counted and the percentage of live cells was calculated as a measure of viability. Each section was counted 3 times as there were more than 100 cells in each section and the counts were averaged.

Statistical analysis

Because the ranges of scores differed among the different scoring systems, the scores were expressed as a percentage of the maximum score according to the method of Sabo *et al.* [25]. For radiographic and histological evaluation, we used the non-parametric Mann-Whitney U-test. Minor local complications were not considered

to represent a reason for withdrawing a single animal, or a group, from the experimental trial. Severe infection or wound dehiscence, which required additional treatment or led to premature death, were defined as individual stopping rules. Viability data of the chondrocytes before and after culture were subjected to arcsin 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

Experiment 1; Reconstruction with autologous frozen whole-knee joint in a rabbit model

Radiological evaluation

Bone union in the L- and C-groups progressed over time. Callus formation was detected in all rabbits after 4 weeks, and osteotomy lines were no longer visible in 3 of 5 rabbits of both groups at 12 weeks (Fig.1). No fractures, graft shortening, or collapse of the subchondral bone were seen. According to the ISOLS graft evaluation system, no differences between the total score of the L-group and that of the C-group were observed at any of the follow-ups (Table 1).

Histology of bone healing

There was no difference between the C- and L-groups in the score of Heiple et al at any time points (Table 1). Total score increased in a time-dependent manner in both groups and was almost the same for both groups at 12 weeks, although the C-group had a better total score than the L-group at 4 weeks. At 4 weeks, callus formation originated mainly from host bone in all rabbits of the L- and C-groups. The bone marrow was dead throughout the joint autograft and it had begun to be replaced by new tissue in all

rabbits in the L- and C-groups, although regeneration took place more rapidly in the C-group. At 8 weeks, the bone union process had progressed gradually in both groups. The regeneration of bone marrow, which appeared to have invaded from the host bone or the surrounding tissue through the junction, progressed gradually in both groups. At 12 weeks, osteochondral union or bone union was achieved in all rabbits of the C-group. All rabbits in the L-group also achieved union, except for one rabbit that apparently had a nonunion (Fig. 2A, B).

Histology and histochemistry of articular cartilage

According to the Mankin score, the total score of L-group was significantly higher than that of group C at every time point (Table 1). No difference was seen in histological findings of cartilage between the femoral side and tibial side. At 4 weeks, the thickness and surface regularity of the articular cartilage was normal in four of five rabbits of both the C- and L-groups (Fig. 3A, B). However, no nuclear staining of the chondrocytes was observed in any rabbit of the L-group suggesting chondronecrosis had occurred. Furthermore, cartilage matrix did not stain with safranin-O in four of five rabbits of the L-group. For these reasons, the Mankin score in the L-group was significantly higher compared with the C-group. In three rabbits at 8 weeks and two rabbits at 12 weeks of C- group, the cartilage architecture was normal. There were no osteoarthritic changes besides surface irregularity in all rabbits of the C-group at 8 and 12 weeks. Cartilage deterioration was minimal and not progressive in the C-group (Fig.3 C, E). In the L-group, no rabbits had live chondrocytes or proteoglycan content at 8 and 12 weeks. Surface irregularities occurred in all rabbits and clefts involving the radial and calcified zone were observed in two of five rabbits, respectively, in the L-group at 8 and 12 weeks (Fig. 3 D, F).

Histology of other joint components

There was little difference between the C- and L-groups in the findings for other joint components such as menisci and ligaments. The menisci and ligaments appeared grossly normal with no sign of tear in all rabbits of both groups. Histological findings were almost identical in the menisci and ligaments of both groups. At 4 weeks, the freezing process was observed to have killed all cells in the menisci. At 8 and 12 weeks, the menisci were being repopulated with spindle-shaped fibrous cells from the synovium (Fig. 2C). Living cells in the ligaments were not detected at 4 weeks in the L-group. At 8 and 12 weeks, the recovery of cellularity progressed from the superficial layer bordering the synovial sheath (Fig. 2D). The collagen structure of the ligaments was almost the same in both groups.

Experiment 2; Vitrification of articular cartilage discs for cryopreservation.

Histological, histochemical and ultrastructural findings of vitrified articular cartilage

Histological and histochemical findings were similar among all the groups. The matrices in the V- and N-groups were stained with safranin-O as well as those in the F-group. In the F-group, ultrastructure appeared normal. The chondrocytes had large round shaped nuclei and some cytoplasmic organelles that were enclosed by an intact plasma membrane with cytoplasmic processes (Fig. 4A). The extracellular matrix was homogeneous with randomly arranged collagen fibers of varying diameters. In the N-group, all chondrocytes had destructive changes in their ultrastructure (Fig. 4C). Many vacuoles were present in the cytoplasm. The cytoplasmic membrane was not continuous and there were no normal appearing cytoplasmic organelles. On the other hand, the extracellular matrix appeared essentially the same as in the F-group. In the V-group, some chondrocytes had various degrees of ultrastructural changes, such as vacuolation of the cytoplasm, disruption of the cytoplasmic membrane, or crenate nuclei.

However, many chondrocytes preserved normal appearing nuclei, cytoplasmic organelles, and a continuous cytoplasmic membrane (Fig. 4B). No difference was observed in the extracellular matrix structure compared with the F-group.

Viability of chondrocytes

Chondrocyte viability is shown in Fig. 5. The mean percentages of chondrocyte viability before and after 24-h culture in group F were 75.9% and 67.2%, respectively. No viable chondrocytes were detected in samples from the N-group except for 1 sample before culture and 2 samples after 24 h of culture. In these latter 3 samples, blue staining chondrocytes, which indicates viable cells, comprised < 3% of all cells. The mean viability of chondrocytes before and after 24-h culture in the N-group was 0.75%. The mean percentages of viable chondrocytes before and after 24-h culture in the V-group were 65.3% and 61.8 %, respectively. Although the viability rate of the chondrocytes in the V-group was significantly lower ($P<0.01$) than that of the F-group, the percentage of viable chondrocytes vitrified with cryoprotectants was more than 85% of that compared to viable cells in fresh cartilage.

Discussion

Our investigation found that frozen grafts were incorporated into host bone and excellent remodeling occurred in frozen bone as well as in control bone. On the other hand, cartilage deterioration was significantly more extensive in the frozen group than in the control group, although the structures of the other joint components were well-preserved.

Various types of whole joint allografts have been investigated in experimental animals and in humans [1,6,7,11,15,26,28,38,39]. In many of the whole joint re-implantation attempts, the surgical technique was difficult and the success of

re-implantation was threatened by the loss of bone fixation and infection [6,26,28]. In this study, we adopted a new freezing method called *in situ* pedicle freezing that was reported in a clinical study [37]. Our whole-knee joint graft model suffered from no fractures, shortening, or failure of fixation in either group. In *situ* freezing simplifies the surgical procedures and increases the ease of obtaining good stability and alignment of the joint without requiring extensive tissue dissection.

Bone union, or the remodeling of frozen osteoarticular allografts, was delayed as compared with fresh autografts in animal models [10,33]. Our histomorphological findings showed that cortical bone union in both groups had occurred in a similar manner and both groups had good remodeling. Frozen autografts may be superior to frozen allografts with respect to incorporation into host bone and remodeling because frozen allografts induce an allogeneic immune response [33]. We found that bone marrow was dead throughout the joint autograft and that callus formation originated mainly from the host bone in both the control and frozen groups. These findings suggest that all grafts were affected by a deprivation of blood supply. The influence of treatment with LN_2 may thus be less than the effect of blood supply deprivation.

When whole-knee joints were transplanted with osteotomies on both sides of the knee in dogs, destructive changes of the joint were observed not only with frozen allografts but also with fresh autografts [11]. In those models, surgical injury and delay of revascularization to the joint were major factors in joint deterioration. In our control model, chondrocyte cellularity and safranin-O staining did not decrease over time. Isolation of the joint with a one-sided osteotomy might cause minimal deprivation of the vascular supply to the joint. In the group treated with LN_2 (L-group), intact chondrocytes were not detected in the lacunae and safranin-O staining was reduced after 4 weeks. The histological structure of the cartilage was sequentially disturbed at 8 and 12 weeks. Since rapid cooling with LN_2 had little effect on the matrix [8], cryoinjury to

312 the chondrocytes that secrete cartilage matrix was the most important factor for
313 progression of cartilage deterioration in the L-group. A loss of chondrocyte viability
314 caused rapid reduction of proteoglycan content in the matrix.

315 Histological evaluation of the menisci and ligaments found little damage to these
316 structures in both groups. Although cellularity was decreased after surgery, a gradual
317 increase in cellularity was observed in both groups. These joint components were
318 studied in frozen allograft models and it was found that function was preserved and the
319 cell population was recovered after transplantation [12,19,24]. Therefore, it appears that
320 the menisci and ligaments have the potential to recover from the treatment with or
321 without LN2 even in case of whole-knee joint graft model.

322 Our findings suggest that cartilage must be protected from cryoinjury during LN_2
323 treatment. Vitrification has been reported to provide effective preservation for
324 monocytes, ova, pancreatic islets, and vascular grafts [16,29,32,34]. Vitrification of
325 cartilage has been studied with a protocol using the VS55 formulation (2.2 M propylene
326 glycol, 3.1 M formamide, and 3.1 M Me_2SO) [8,30,31]. Rabbit cartilage could be
327 preserved *in vitro* with more than 80% cell viability [31]. In an *in vitro* assay of bovine
328 cartilage [8], VS55 treatment retained an average of 51% viable chondrocytes after
329 rapid freezing. These results were considerably better than those obtained using a
330 conventional cryopreservation protocol. However, all of these vitrification protocols are
331 complicated and require more than 90 minutes for the stepwise addition and removal of
332 cryoprotectants. We developed a new vitrification protocol by modifying a vitrification
333 method utilized for embryo cryopreservation. Though the ultrastructure appeared
334 normal in the Fresh group, the mean percentage of chondrocyte viability before culture
335 in this group was 75.9%. This percentage is lower than that reported in other papers [8],
336 which is probably caused by differences in the methods of viability assessment.
337 Conversely, the mean percentage of viable chondrocytes in the vitrificaton group was

65.3% before culture and 61.8% after 24 hours of culture. Vitrified cartilage cell viability was more than 85% compared to fresh cartilage. This data is comparable to that of other vitrification protocols in *in vitro* models, although our protocol is very simple in comparison. We employed ethylene glycol as the cryoprotectant and added it in two sequential, 2-minute steps. One of the major factors determining the permeation rate of a cryoprotectant into a cell is its molecular weight (MW). Because ethylene glycol (MW 62.1) is smaller than DMSO (MW 78.1) or glycerol (MW 92.1), which are the most popular cryoprotective agents for cryopreservation of cartilage or chondrocytes, ethylene glycol may permeate more rapidly into cartilage. Rapid permeation may be one reason why our protocol achieved good results.

In reconstructions using frozen autografts that contain cancerous tissue, if the tumor has invaded the cartilage, we cannot use this vitrification method because it will also preserve the viability of some tumor cells that might be present in the graft. However, if the tumor is clearly separate from the cartilage, it may be possible to protect the cartilage selectively from cryoinjury by applying vitrification solution to only the cartilage surface. Further research is required to determine whether we can selectively protect cartilage from cryoinjury.

Our vitrification protocol can be utilized for the cryopreservation of osteochondral allografts. One of the most important factors concerning a successful clinical outcome after transplantation of osteochondral allografts is the viability of cartilage. Since our vitrification protocol is very simple and does not need special equipment, our protocol may be able to preserve viable cartilage easily. The present data showed that our vitrification protocol is effective to protect cartilage from cryoinjury in an *in vitro* study using a rabbit model. However, to utilize this vitrification method in humans, further investigations are necessary to determine whether we can protect human cartilage from cryoinjury since it is thicker than rabbit cartilage.

In conclusion, cryoinjury to chondrocytes of articular cartilage causes osteoarthritic changes following joint reconstruction with frozen osteoarticular grafts. To prevent osteoarthritic changes, articular cartilage needs to be protected from cryoinjury during LN_2 treatment. Our vitrification method was shown to be effective *in vitro* for protecting cartilage from cryoinjury. If the cartilage can be selectively preserved from cryoinjury with vitrification, autologous frozen whole joint grafts could become an ideal approach for joint reconstruction. Furthermore, vitrification may be useful for storing living cartilage for allografts.

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Figure legends

Figure 1. Radiographs show the lateral views of the hind limbs in the control group (A, B, C) and in the liquid nitrogen-treated group (D, E, F). Radiographs were taken at 4

494 weeks (A, D), 8 weeks (B, E), and 12 weeks (C, F). Callus formation was seen after 4
495 weeks and the osteotomy line was no longer visible in 3 animals of both groups at 12
496 weeks.
497
498 Figure 2. Photograph of the osteotomy site (A), bone marrow (B), menisci (C) and
499 ligaments (D) from the liquid nitrogen-treated group at 12 weeks. Bone union was
500 observed (A) and dead narrow was replaced by fibroblastic cells, haematopoetic cells,
501 and new fatty marrow cells (B). Menisci (C) and ligaments (D) were repopulated with
502 spindle-shaped fibrous cells (hematoxylin and eosin stain, ×40 (A), ×100 (B-D)).
503
504 Figure 3. Photograph of articular cartilage from the control group (A, C, E) and from
505 the liquid nitrogen-treated group (B, D, F). Cartilage was evaluated at 4 weeks (A, B),
506 8 weeks (C, D), and 12 weeks (E, F). Cartilage deterioration was not progressive in the
507 control group, but regressive changes progressed over time in the liquid
508 nitrogen-treated group (saffranin-O stain, ×100).
509
510 Figure 4. Electron micrographs of chondrocytes from fresh (A), vitrification (B) and
511 non-vitrification (C) cartilage after 24 h of culture. Bar represents 1 μm. Fresh
512 chondrocytes had normal appearing nuclei and cytoplasm. Chondrocytes from the
513 vitrification group also had a normal appearance with round nuclei and an intact
514 plasma membrane. Chondrocytes from the non-vitrification group appeared disrupted
515 with a heavily vacuolated cytoplasm and irregular nuclei.
516
517 Figure 5. Viability of chondrocytes from each experimental group. Data were
518 expressed as the mean ± S.E.M. * Significantly different using post hoc Fisher's
519 protected least significant difference test ($P < 0.05$)

Table 1. Outcome measures for the liquid nitrogen-treated (L) and control (C) groups

	4 weeks			8 weeks			12 weeks		
	C	L	P Value	C	L	P Value	C	L	P Value
ISOLS score	93.3 ± 2.3	89.2 ± 5.6	0.25	93.3 ± 3.7	92.5 ± 3.5	0.92	95.8 ± 5.1	90.8 ± 6.8	0.17
Heiple score	46.7 ± 14.0	35.0 ± 18.1	0.13	65.0 ± 7.0	61.7 ± 13.9	0.74	73.3 ± 9.1	73.3 ± 6.9	0.9
Mankin score	30.0 ± 15.5	51.4 ± 7.8	0.014	12.9 ± 9.3	75.7 ± 8.1	0.009	28.6 ± 19.6	70.0 ± 7.8	0.007

* The values are expressed as a percent of the maximum. The data are listed as the mean and the standard deviation.

* The Mankin scores were significantly different between the C- and L-groups at 4, 8, and 12 weeks using the Mann-Whitney U-test ($P < 0.05$).

Figure 1.

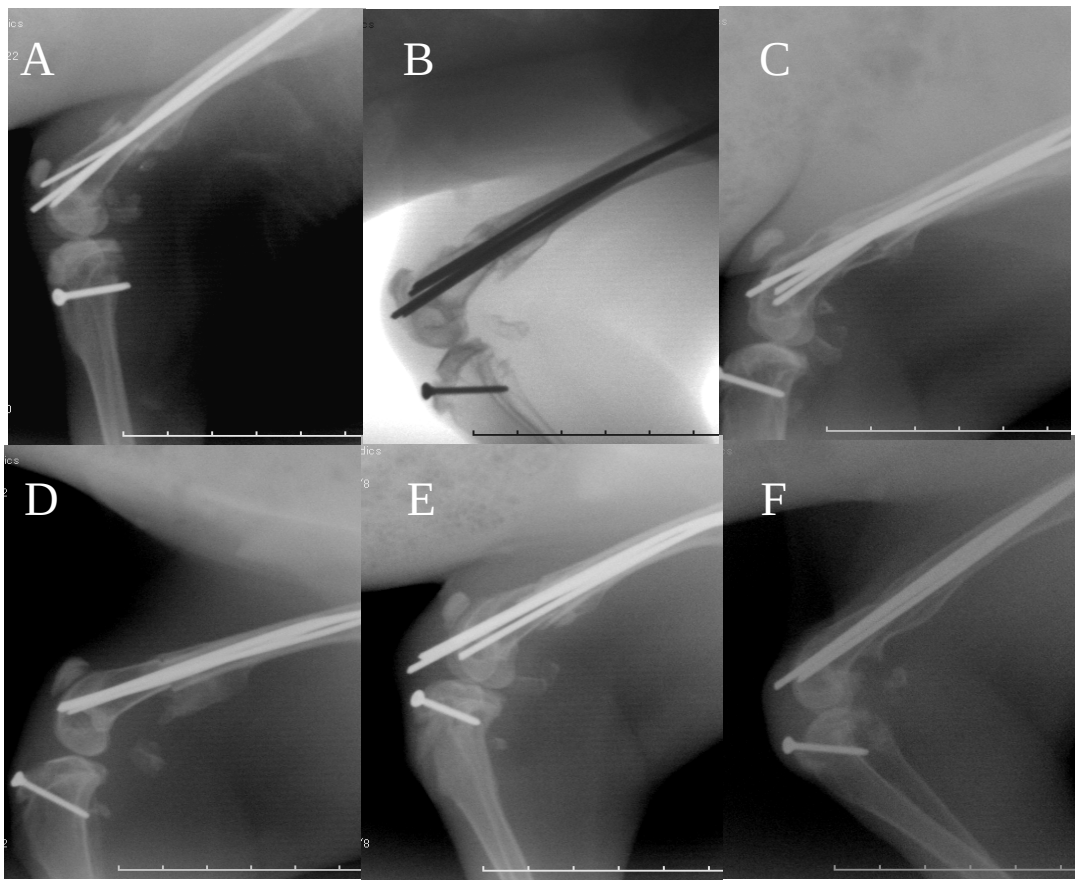
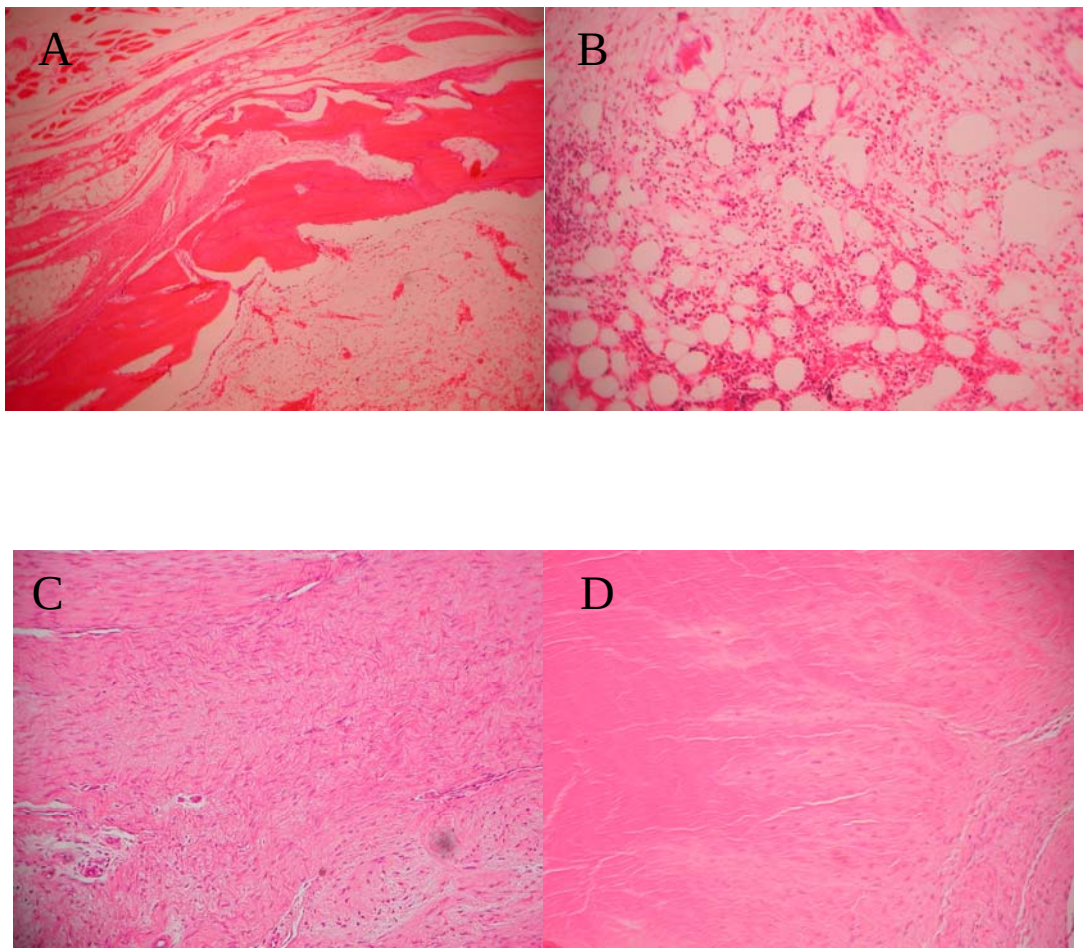


Figure 2.



Influence of freezing with liquid nitrogen on whole-knee joint grafts and protection of cartilage from cryoinjury for limb-saving surgery: Experimental study in rabbits

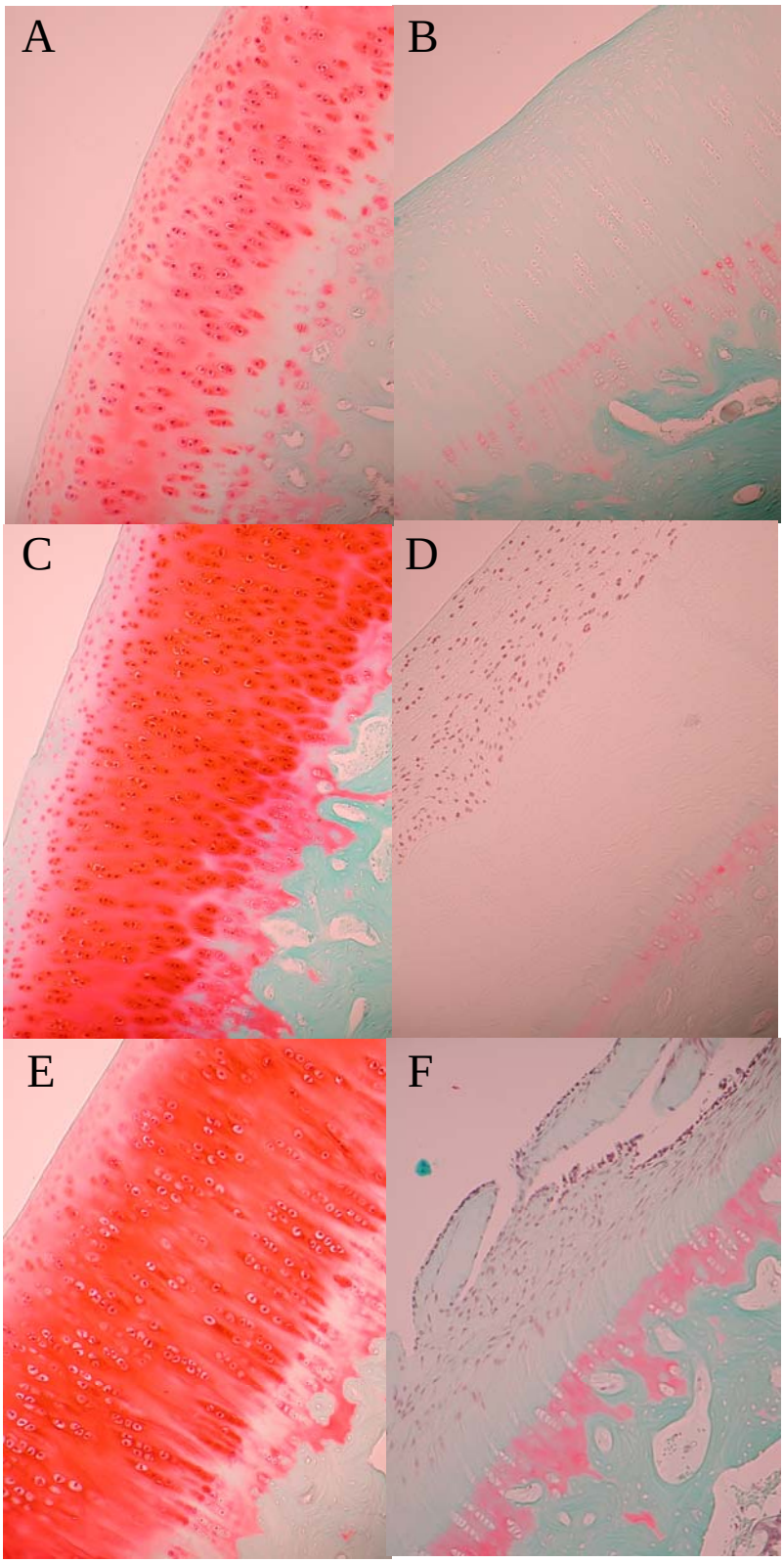
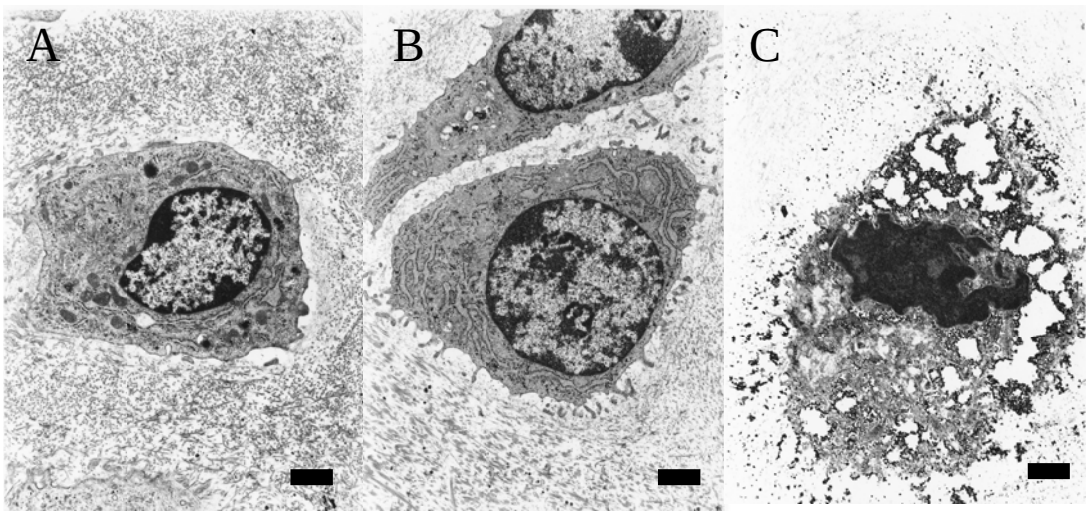
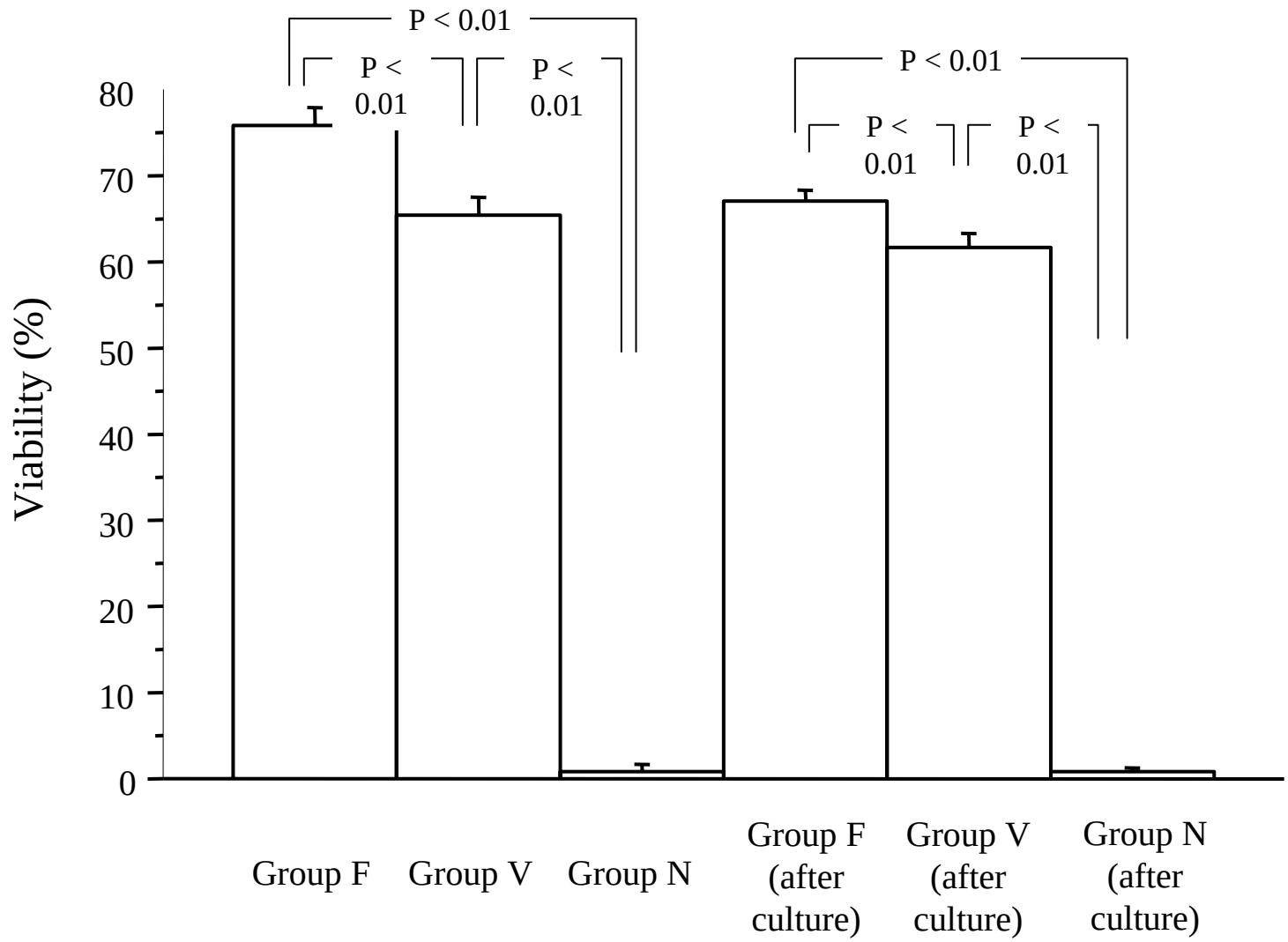


Figure 4.




P < 0.01
P < 0.01
P < 0.01
P < 0.01
P < 0.01
P < 0.01
Viability (%)
80
70
60
50
40
30
20
10
0
Group F
Group V
Group N
Group F (after culture)
Group V (after culture)
Group N (after culture)