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Vitreous Preservation of Rabbit Articular Cartilage

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ABSTRACT

The growing need for improved methods of viable tissue cryopreservation has stimulated debate regarding the relative merits of traditional freezing methods versus ice-free vitrification methods. Articular cartilage has proved refractory to satisfactory cryopreservation using conventional freezing methods. Therefore, full-thickness rabbit femoral head articular cartilage was used to compare a freezing method of cryopreservation and an ice-free, vitrification method of cryopreservation with fresh controls *in vitro*. Chondrocyte viability was determined *in vitro* using vital fluorescent staining with calcein-acetoxymethyl ester and an oxidation–reduction assay with alamarBlue™ (Accumed International, Westlake, Ohio). Cryosubstitution with 1% osmium tetroxide in 100% methanol was used to detect ice during cryopreserved storage. Cryosubstitution studies of frozen and vitrified articular cartilage pieces revealed negligible ice in the vitrified specimens and extensive ice formation in frozen specimens with the exception of the articular surface. Frozen specimens retained <13% viability. In contrast, postvitrification tissue had >80% of control viability. The distribution of ice observed in cartilage cryopreserved by freezing suggests that ice formation was the cause of poor cryopreserved cartilage viability reported in the literature. The results demonstrate the feasibility of vitrification as a storage method for cartilaginous tissues.

INTRODUCTION

FRESH OSTEOCHONDRAL ALLOGRAFTS have proven to be effective and functional for transplantation. However, the limited availability of fresh allograft tissues necessitates the use of osteoarticular allograft banking for long-term storage.[1–4] Simple freezing of cells or tissues without cryoprotectants and a cryopreservation protocol results in nonviable, nonfunctional materials. Little advance was made in the field of cryopreservation until Polge et al.[5] discovered the cryoprotective properties of glycerol. Subsequently, Lovelock and Bishop[6] discovered that dimethyl sulfoxide (DMSO) could also be used as a cryoprotectant. Since the discovery of these cryoprotective agents, cryoprotection during freezing and thawing of biological materials has become established, and many other cryoprotectants have been identified. Isolated chondrocytes in suspension, in common with many other cell types, can be preserved using conventional cryopreservation methods involving freezing. In such methods the cells are concentrated and vitrified in ice-free channels between regions of extracellular ice; however, chondrocytes embedded in their natural matrix are extremely difficult to preserve by similar freezing methods, presumably because the cells cannot move away from forming extracellular ice and inadequate amounts of cryoprotectant penetrate the cells to prevent intracellular ice formation. Conventional cryopreservation using freezing protocols typically result in death of 80–100% of the chondrocytes. Studies using a variety of animal articular cartilage models[2,3,7,8] and human cartilage biopsies[9] have revealed no more than 20% chondrocyte viability following conventional cryopreservation procedures employing

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either DMSO or glycerol as cryoprotectants. Ohlendorf et al.[3] used a bovine articular cartilage, osteochondral plug model to develop a clinical cryopreservation protocol. This protocol employed a slow rate of cooling and 8% DMSO as the cryoprotectant. They observed loss of viability in all chondrocytes except those in the most superficial layer near the articular surface.

In a recent editorial the need for an alternative to cryopreservation by freezing was emphasized.[10] Ice can be avoided by vitrification. Vitrification refers to the physical process by which a concentrated solution of cryoprotectants solidifies during cooling without crystallization. The extremely viscous supercooled liquid, called glass, retains the normal molecular and ionic distributions of the liquid state, but it is usually considered to be a solid.[11] Vitrification has been shown to provide effective preservation for a number of cells, including monocytes, ova, early embryos, and pancreatic islets.[12–15] Our laboratory has developed an ice-free cryopreservation procedure using vitrification for tissues.[11,16,17] This method was based upon a vitrification formulation that was originally designed for organ preservation.[12,18] Studies with vitrified vascular grafts have demonstrated preservation of smooth muscle cell function (>80%) and normal performance as vascular grafts.[16,17,19] However, demonstration of *in vivo* function of vitrified organs has yet to be accomplished.

The major objective of this study was to test our baseline vitrification procedure on a tissue that has traditionally been extremely difficult to cryopreserve by conventional freezing methods. A second goal was to formally establish a link between the location of ice and cell viability in frozen cryopreserved cartilage.

MATERIALS AND METHODS

Surgical methods

Male New Zealand white rabbit full-thickness (~0.6-mm) femoral head cartilage was obtained using sterile technique following anesthesia induction with ketamine and xylazine and sacrifice using an intravenous overdose of pentobarbital (90–120 mg/kg). Cartilage specimens were prepared as either small (full thickness × 1.0 mm × 1.0 mm) or large (full thickness × 2–4 mm × 2–4 mm) samples. All animal care and handling complied with the "Principles of Laboratory Animal Care" as formulated by the National Society for Medical Research and the guidelines of the National Research Council.[20]

Conventional cryopreservation

Cartilage specimens were cryopreserved in polyethylene vials using a slow rate (−1°C/min) of cooling with 1.4 *M* DMSO in Dulbecco's Modified Eagle's Medium (DMEM) culture medium plus 10% fetal bovine serum from 4°C to −80°C. The cryopreserved tissue specimens were then stored at −160°C in vapor-phase nitrogen for a minimum of 24 h. Thawing was accomplished in two steps. In the first step the specimens were placed at room temperature and warmed from −160°C to −100°C at ~20°C/min, and in the second step the specimens were immersed in a 37°C water bath and warmed at ~50°C/min until all the ice had disappeared, whereupon the containers were transferred to an ice-bath for elution of the cryoprotectant. This was achieved in one step in which the tissue samples were transferred to DMEM. Viability assessments were initiated within 1 h of completion of the rewarming and cryoprotectant elution protocol.

Vitrification protocol

The cartilage specimens were gradually infiltrated with an 8.4 *M* vitrification solution consisting of 3.10 *M* DMSO, 3.10 *M* formamide, and 2.21 *M* 1,2-propanediol in EuroCollins solution at 4°C.[17] Precooled vitrification solution (4°C) was added in six sequential, 15-min steps. After addition of the final vitrification solution, the cartilage was placed in glass scintillation vials (diameter × height, 25 mm × 60 mm) containing 2 mL of precooled vitrification solution. The top of the vitrification solution was then covered with 0.7 mL of 2-methylbutane (isopentane; freezing point, −160°C; density, 0.62) at 4°C to prevent direct air contact. A thermocouple was inserted into a separate dummy sample of the same vitrification solution, and its output was monitored via a digital thermometer throughout the cooling process. Sam-

ples were cooled rapidly (43°C/min) to −100°C by placing the samples in a precooled bath containing isopentane in a −135°C mechanical storage freezer. Upon achieving −100°C the specimens were removed from the bath and stored at −135°C in the mechanical storage freezer, which resulted in slow cooling (3°C/min) to −135°C. The samples were at −135°C for a minimum of 24 h. Vitrified cartilage was rewarmed in two stages; first, slow warming to −100°C (~30°C/min) at the top of the mechanical storage freezer, and then rapidly warmed to melting (~225°C/min) in a 30% DMSO in water bath at room temperature. After rewarming, the vitrification solution was removed in seven sequential 15-min steps at 4°C into DMEM culture medium. In the seven steps the 8.4 *M* cryoprotectant components of the vitrification solution were decreased from 8.4 *M* in EuroCollins Solution → 4.2 *M* + 300 m*M* mannitol → 2.1 *M* + 300 m*M* mannitol → 1.05 *M* + 300 m*M* mannitol → 0.525 *M* + 300 m*M* mannitol → 0 *M* + 300 m*M* mannitol → Eurocollins Solution → DMEM.[16] Viability assessments were initiated within 1 h of completion of the rewarming and cryoprotectant elution protocol.

Viability assessment 1

Small full-thickness cartilage specimens were incubated under cell culture conditions in media containing alamarBlue™ (Accumed International, Westlake, OH). Samples were analyzed every hour for 6 h. The alamarBlue assay utilizes a water-soluble fluorometric viability indicator based on the detection of metabolic activity, specifically, an oxidation–reduction indicator that both fluoresces and changes color in response to chemical reduction of the growth medium caused by cell metabolism. Samples were read on a spectrofluorometer at 590 nm. The data are expressed as relative fluorescent units per milligram dry weight of tissue.

Viability assessment 2

Vital fluorescent staining with calcein-acetoxymethyl ester (calcein-AM) was performed on large full-thickness specimens to determine chondrocyte viability in the surface layers of the cartilage. After staining, the cartilage slices were mounted on slides and viewed using a fluorescent microscope. The viable cells with intact membranes metabolize calcein-AM and fluoresce green. The fluorescent intensity was measured, and the data are normalized as a percentage of fresh tissue relative fluorescent units.

Histology of cryosubstitution

Cryosubstitution is a method for demonstrating the presence or absence of ice during cryopreserved storage.[21] Cryosubstitution was performed on large full-thickness cartilage specimens using chilled (−90°C) 1% osmium tetroxide in 100% methanol in high-density polyethylene scintillation vials containing cryopreserved specimens at −90°C. The tissues were dehydrated by replacing the frozen or vitrified water with cryosubstitution medium over a period of several days at −90°C. The heat-sink and vials were then placed in a −20°C freezer overnight, followed by 4°C for 1 h, and then finally brought to room temperature. This gradual warming of the tissue and cryosubstitution media assures complete osmium tetroxide tissue fixation. Finally, these tissues were transferred to 100% acetone, infiltrated with Araldite® resin (Polysciences, Inc., Warrington, PA) and polymerized, sectioned, stained with toluidine blue, and viewed by light microscopy. Selected regions of representative blocks were then sectioned (75 nm) for transmission electron microscopy using a diamond knife on a Reichert OMU2 ultramicrotome. The sections were double-stained using uranyl acetate followed by lead citrate and viewed in a JEM-1210 transmission electron microscope (JEOL USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV.

Statistical methods

All experiments were repeated at least three times, and statistical differences were assessed by one-way analysis of variance. Values of $p < 0.05$ are regarded as statistically significant.

RESULTS

Cryosubstitution studies of frozen and vitrified articular cartilage plugs revealed negligible ice in the vitrified specimens (Fig. 1A), and

extensive ice formation, in both the extracellular matrix and deeper lacunae, in frozen specimens (Fig. 1B). Some cell shrinkage was observed in the lacunae of vitrified specimens (Fig. 1A), which is most likely related to high cryoprotectant concentrations. Representative electron micrographs of fresh (Fig. 1C and D) and vitrified, cryosubstituted (Fig. 1E and F) cartilage samples are shown for comparison. Increased vacuolation was evident in some cells deep in the transitional layer of vitrified specimens (Fig. 1F). These vacuoles may represent a low level of intracellular ice formation.

Analysis of variance was performed on the data at each time point in the alamarBlue studies of oxidation–reduction after cryopreservation. The analyses demonstrated that specimens cryopreserved by vitrification were

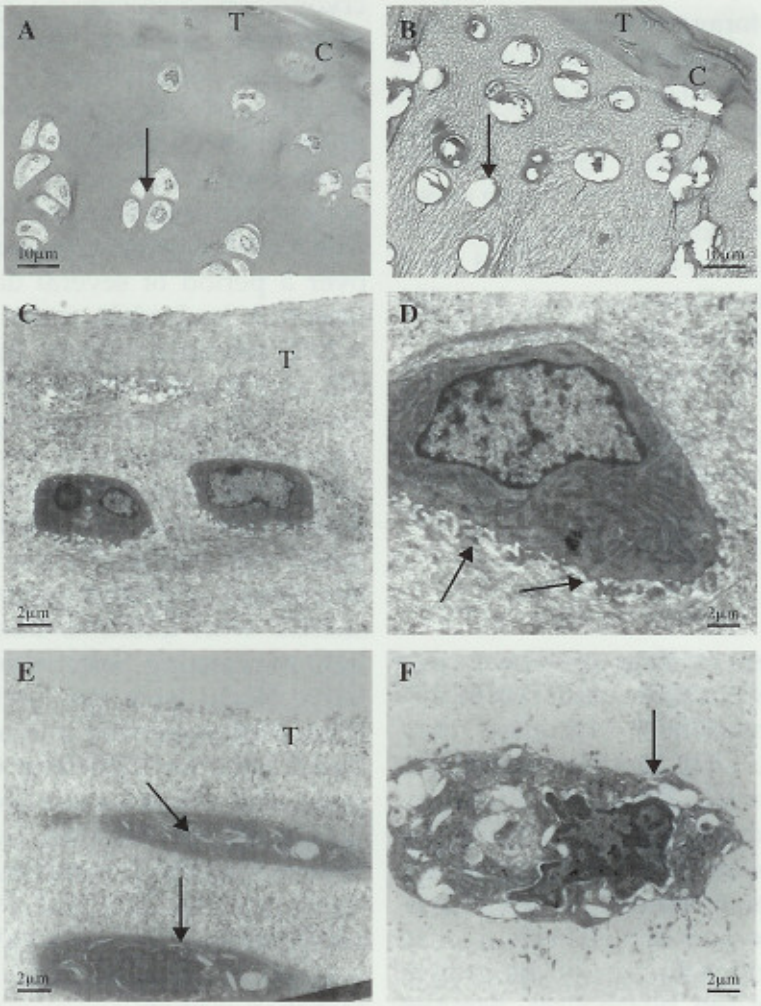

FIG. 1. Light microscopy of vitrified (**A**) and frozen (**B**) cryosubstituted articular cartilage. Cryosubstitution reveals the location of ice domains within cryopreserved tissues as indicated by white spaces in the tissue section. **A:** Vitrified. The matrix shows little ice formation; the tangential layer (T) reveals an elliptical chondrocyte (C) within its lacuna. The deeper chondrocytes (arrow) show some shrinkage and space associated with lacunae, which contain granular material. **B:** Frozen. The chondrocytes within the ice-filled lacunae (arrow) appear totally disrupted; the deep matrix has considerable ice formation. The chondrocytes (C) within the superficial and tangential layer (T) appear to have less ice. ×100. **C–F:** Electron micrographs of fresh (**C** and **D**) and vitrified, cryosubstituted (**E** and **F**) articular cartilage. **C:** The tangential layer (T) is shown with representative chondrocytes clearly visible. ×4,000. **D:** Deeper within the transitional layer, a chondrocyte and its cytoplasmic protrusions (arrows) are shown. ×10,000. **E:** The chondrocytes (arrows) within the superficial layer (T) are elliptical with few vacuoles. The intercellular substance appears slightly condensed. ×5,000. **F:** Chondrocytes deep within the transitional layer appear more heavily vacuolated (arrows) than those within the superficial layer. ×10,000. These vacuoles may represent a low level of intracellular ice formation.

significantly more metabolically active than specimens cryopreserved by freezing ($p < 0.0001$, Fig. 2). Fluorescent microscopy studies using the vital stain calcein-AM demonstrated small numbers of viable cells in the first 30–50 μm below the articular surface of frozen specimens (Fig. 3C). In contrast, the majority of cells were viable in fresh and vitrified specimens (Fig. 3A and B). Quantitative analysis of the relative fluorescent intensity readings for calcein-AM (mean ± 1 SEM) demonstrated no significant differences in intracellular esterase activity in fresh and vitrified specimens (Fig. 4). In contrast, cartilage cryopreserved by freezing retained significantly less intracellular esterase activity than either fresh or vitrified specimens ($p < 0.05$, Fig. 4). Thus, two different assays of cell viability, measuring oxidation–reduction and intracellular esterase activity, respectively, demonstrated that rabbit cartilage specimens cryopreserved by vitrification retained >80% of values obtained for fresh samples (Figs. 2 and 4). In contrast, frozen samples retained <13% of fresh control viability by each assay (Figs. 2 and 4).

These studies combine to demonstrate that the vitrification process results in ice-free preservation (Fig. 1A) of full-thickness rabbit articular cartilage and that >80% of the cells were viable and metabolically active following rewarming. Frozen tissues contained ice within both the cells and the matrix (Fig. 1B), with the exception of the articular surface, where some viable cells were observed by fluorescent microscopy (Fig. 3C). Overall cell survival was <13% in frozen specimens (Figs. 2 and 4).

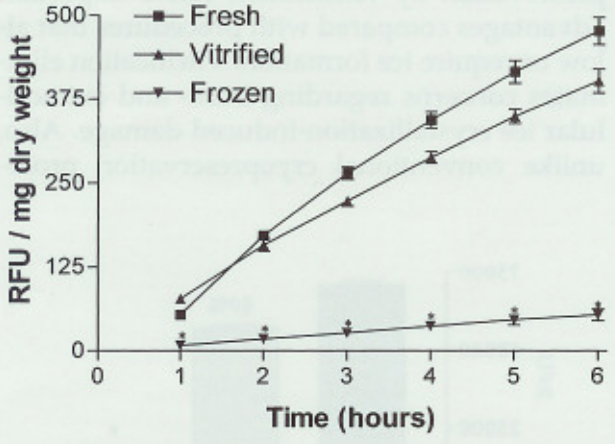

FIG. 2. Viability of articular cartilage using alamarBlue as a measure of metabolic activity. Data are expressed as the mean ± 1 SEM of 10 samples. At each time point one-way analysis of variance using the Kruskal–Wallis test revealed significant treatment effects: $^{*}p < 0.0001$. Dunn's multiple comparison test showed that there were no significant differences between fresh and vitrified groups, while there were significant differences between fresh and frozen and between vitrified and frozen groups at all time points. RFU, relative fluorescence units.

DISCUSSION

In this study the baseline vitrification cryopreservation procedure, previously employed with cardiovascular tissues, was tested on articular cartilage. The results confirmed previous studies indicating that articular cartilage is extremely difficult to cryopreserve by conventional freezing methods employing 1–2 *M* DMSO. Frozen specimens retained <13% viability (Figs. 2 and 4). The vitrified specimens, preserved with an 8.4 *M* cryopreservation solution consisting of a mixture of DMSO, formamide, and propanediol, retained >80% of control viability (Figs. 2 and 4). Recently Jomha et al.[22] demonstrated that increasing the DMSO concentration to 6 *M* can result in higher overall cell survival (40%) after cryopreservation. It is not clear from their study whether vitreous or frozen cryopreservation was achieved at 6 *M* DMSO. Vitrification was demonstrated in the present study by the absence of ice in cryosubstituted specimens (Fig. 1). It is possible that some ultrastructurally detected vacuolation in cryosubstituted specimens (Fig. 1F) was due to ice formation. This could indicate that the vitrification procedure employed may need further improvement, particularly for larger mammalian tissue models and human specimens in which the cartilage is much thicker than in the rabbit. However, at this time we have no formal proof that the vacuolation observed in chondrocytes deep in the transitional layer is actually due to ice formation.

A second goal of this study was to determine the location of the ice in cartilage cryopreserved by freezing. Ice was observed in all regions of the cartilage specimens except the most superficial layer beneath the articular surface (Fig. 1B). Small numbers of vital-stained, viable cells

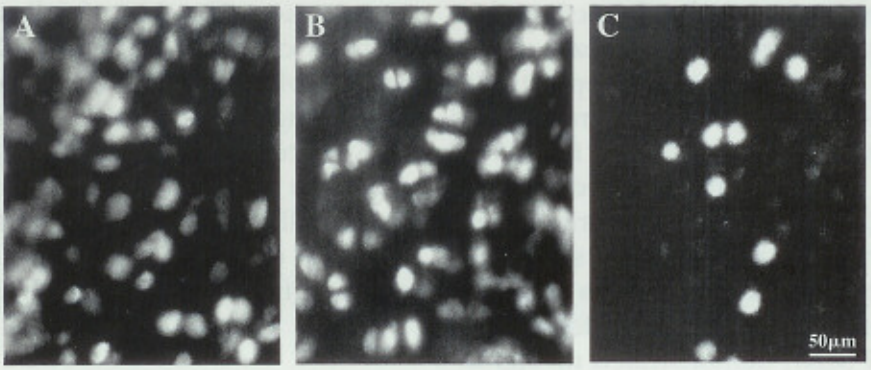

FIG. 3. Representative fluorescent images of (A) fresh, (B) vitrified, and (C) controlled-rate frozen articular cartilage. Samples were screened for viability using calcein-AM (which measures intracellular esterase activity). The images show the live, green cells.

were observed in this region by fluorescent microscopy (Fig. 3C).

There have been several reports that cartilage cryopreserved by freezing results in survival of cells at the articular surface.[3,7] Recently, Muldrew et al.[23] demonstrated improved results using a step-cooling cryopreservation protocol, but cell survival post-transplantation was poor, and again there was significant loss of cells in the midportion of the cartilage. The reason for lack of cell survival deeper than the superficial layers of articular cartilage is most likely multifactorial and related principally to heat and mass transfer considerations.[24] Surface cells freeze and thaw more rapidly than cells located deep within the matrix, and the opportunity for the more superficial cells to lose water by osmotic dehydration is greater. Osmotic dehydration occurs because the ice initially forms in the solution surrounding the cartilage specimen. As ice forms the electrolyte concentration in the surrounding solution increases, resulting in an osmotic imbalance in response to which the more superficial cells lose water. However, the deeper cells lose water more slowly. This phenomenon could result in a greater opportunity for ice to form within cells deeper within the articular cartilage. Furthermore, typically employed concentrations of DMSO (8–20%) may not penetrate adequately to limit intracellular ice formation.[22] In a similar manner it is anticipated that modifications to the cryoprotectant loading procedures employed for cartilage vitrification may be required as further research progresses from using relatively thin rabbit cartilage to large mammal models in which the cartilage may be almost an order of magnitude thicker.

We are aware that other factors, in addition to ice formation, may have biological consequences during freezing. Several hypotheses have been published on alternative mechanisms of freezing-induced injury,[24,25] but our own experiences with mammalian tissues concur with others that the disadvantages of conventional cryopreservation revolve primarily around ice formation.[11,16,17,26,27]

The present data support a strong case for ice-free cryopreservation by vitrification. Cryopreservation by vitrification offers important advantages compared with procedures that allow or require ice formation. Vitrification eliminates concerns regarding intra- and extracellular ice crystallization-induced damage. Also, unlike conventional cryopreservation proce-

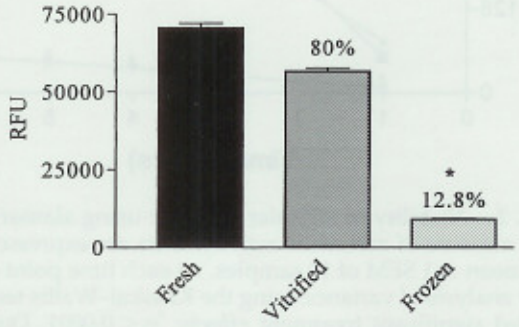

FIG. 4. Quantitative analysis of intracellular esterase activity employing calcein-AM. Data are expressed as mean ± 1 SEM of four experiments. $^{*}p < 0.05$ by one-way analysis of variance using the Kruskal–Wallis test and Dunn's multiple comparison post-test. RFU, relative fluorescence units.

dures that employ freezing, vitrification does not require controlled cooling and warming at optimum rates—cooling and warming need only be rapid enough to prevent ice formation, and this can generally be achieved without specialized equipment.[11,21] However, while vitreous cryopreservation provides superior preservation compared with conventional freezing methods in relatively small model systems, such as the 2-mL cartilage model employed in this report, cryopreservation of large tissue samples continues to be hampered by thermomechanical constraints. Hence a major hurdle for deployment of vitrification methods is the development of effective rapid cooling and warming techniques for larger specimens (greater than 10 mL in volume) to prevent ice growth. Novel warming devices designed to achieve uniform and high rates of temperature change have been described.[28–30] Successful application of these technologies to the survival of cells and tissues has not yet been reported. An alternative, or even adjunctive, approach involving the use of molecular ice control techniques to prevent the damaging growth of ice crystals during cooling and warming of large specimens has recently been described.[11] The objective is identification of molecules that specifically interact with ice nuclei, resulting in either prevention of ice nucleus development or modification of ice crystal phenotype. These either natural (i.e., antifreeze peptides) or synthetic (i.e., cyclohexanediols) molecules promise to have benefits in both freezing and vitrification preservation protocols.[11]

In conclusion, the results of this study using a rabbit model indicate that vitrification is superior to conventional cryopreservation by freezing. The preservation technology presented here may enable the long-term storage and transportation of living cartilage for repair of human articular surfaces. This cartilage may be in the form of osteochondral allografts[1–4] or, prospectively, tissue-engineered cartilage constructs.[31–33]

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