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*Title Page

Vitrification of intact human articular cartilage

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Abstract

Articular cartilage injuries do not heal and large defects result in osteoarthritis with major personal and socioeconomic costs. Osteochondral transplantation is an effective treatment for large joint defects but its use is limited by the inability to store cartilage for long periods of time. Cryopreservation/vitrification is one method to enable banking of this tissue but decades of research have been unable to successfully preserve the tissue while maintaining cartilage on its bone base – a requirement for transplantation. To address this limitation, human knee articular cartilage from total knee arthroplasty patients and deceased donors was exposed to specified concentrations of 4 different cryoprotective agents for mathematically determined periods of time at lowering temperatures. After complete exposure, the cartilage was immersed in liquid nitrogen for up to 3 months. Cell viability was 75.4 ± 12.1% determined by membrane integrity stains and confirmed with a mitochondrial assay and pellet culture documented production of sulfated glycosaminoglycans and collagen II similar to controls. This report documents successful vitrification of intact human articular cartilage on its bone base making it possible to bank this tissue indefinitely.

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Introduction

Osteoarthritis (OA) results in a massive socioeconomic burden with major personal implications[1]. Arthritis is a leading cause of work disability, with an annual economic cost of $4.4 billion in Canada[2] and $40 billion in the USA[3]. Almost 60% of those afflicted will be younger than 65 years of age while it is estimated that a quarter of the world's population over the age of 60 suffer from significant joint pain and disability caused by osteoarthritis (OA), the most common form of arthritis. Thus, prevention and treatment of OA are of paramount importance to society. To date there is no known cure for OA; however, several risk factors have been identified including articular cartilage injury.

Small articular cartilage defects can be treated with various techniques such as drilling[4], microfracture[5], mosaicplasty[6], autogenous chondrocyte implantation[7] and matrix-associated chondrocyte implantation[8] with variable success. Unfortunately, these treatment options are not indicated for larger cartilage defects ultimately resulting in joint instability and the development of OA. Fresh osteochondral allografting was popularized by Gross in 1983[9] and has had reasonable success for large joint defects over long periods of time[10, 11]. As safety concerns arose regarding the transmission of infectious diseases with tissue donation during the 1980s, the attractiveness of this treatment option waned because of the necessity of performing the transplantation within 24-72 hours of death of the donor to maintain chondrocyte viability. This led to the development of hypothermic storage at 4°C. Initially reports indicated that the chondrocytes could survive up to 42 days at this temperature but more recent investigations have demonstrated that these cells begin to deteriorate after 7-14 days at 4°C[12] thereby significantly limiting the application of hypothermic storage as an effective tissue banking method. Therefore, the establishment of a cryopreserved tissue bank of articular cartilage could provide a treatment option for patients with large joint defects while minimizing the concerns regarding infectious disease transmission and improving clinical outcomes by providing a large range of sizes to choose from,

optimizing the operating conditions by allowing this surgery to be planned electively, and possibly matching for blood and HLA typing.

Cryopreservation has been successful for various individual cells[13, 14, 15, 16], including articular cartilage chondrocytes[17, 18], but application to larger tissues has been extremely difficult. Specific to articular cartilage, early attempts at cryopreservation employed standard cryobiological techniques that incorporated controlled ice formation such as the 2-step cryopreservation process[19]. Although some success was achieved, most notably in the sheep model[20], it was not successful when applied to human joint cartilage[21]. Investigation revealed that the ice formation was lethal to cells for various reasons[19, 22] but also altered the structural architecture of the cartilage matrix[23] with important implications for the long term function of the transplanted cartilage[24]. For these reasons, investigators explored vitrification, the formation of an amorphous solid from an aqueous solution without nucleating ice. For the purposes of cryopreservation, practical vitrification is understood to mean an absence of ice during the full cooling and warming cycle[25].

Vitrification typically requires high concentrations of cryoprotective agents (CPAs) and a rapid cooling rate[26] to cool below the glass transition temperature (typically around -130°C) where all biological and chemical activities effectively cease. With the necessary CPA concentrations and cooling rate, the solution becomes sufficiently viscous that a solid forms preventing the formation of damaging ice crystals. Storage using vitrification can be indefinite as the cells do not age – as such they are in suspended animation. Importantly, in larger tissues extremely rapid cooling rates are difficult to achieve due to heat transfer concerns, so higher concentrations of CPAs are required. Fahy has demonstrated that it is possible to vitrify larger biologic tissues[27]. Early success was achieved on intact, full thickness rabbit articular cartilage using a combination solution containing dimethyl sulfoxide (DMSO), formamide and propylene glycol(PG)[28]. Further research using the same three CPAs combined in a vitrification

solution, VS55, was able to vitrify thin bovine articular cartilage[29] and a modified version of this solution was successful on porcine articular cartilage removed from its bone base[30]. Furthermore, Pegg achieved some success using a computerized programmed immersion of thin human articular cartilage (approximately 1mm, removed from the bone) into increasing concentrations of DMSO at increasingly lower temperatures[31]. Despite these partial successes, vitrification of intact, thick (2-4mm) human articular cartilage remained unsolved. Through these published works and our own extensive investigation into the cryopreservation of articular cartilage, it became clear that there are three main obstacles to the vitrification of intact articular cartilage: 1) CPA toxicity to chondrocytes, 2) CPA permeation into the cartilage matrix and 3) interactions between the CPAs.

Our work has focused on overcoming the three main obstacles to the vitrification of articular cartilage. We have investigated individual CPA toxicity to chondrocytes of 5 different CPAs as well as the toxicity interactions between CPAs[32]. We have studied transport in articular cartilage[33] and have determined the permeation kinetics of 4 CPAs into intact articular cartilage including their temperature dependencies[34, 35]. We have also investigated interactions between different CPAs during the process of vitrification[36]. Furthermore we have introduced accurate models for calculating freezing points of CPA solutions[37] . It was our intention to use the information gathered from these studies and combine it using statistical methodology to create a vitrification protocol that would successfully vitrify intact human articular cartilage. We hypothesized that it is possible to vitrify intact human articular cartilage on its bone base by sequentially introducing multiple CPAs at increasingly lower temperatures resulting in excellent cell survival and matrix integrity. To accomplish this, we examined a variety of solution combinations and CPA addition and cooling protocols on human articular cartilage followed by vitrification in liquid nitrogen. Cell recovery was tested using membrane integrity stains, a metabolic assay and pellet culture with matrix production.

Materials and Methods

Articular cartilage was obtained from human knees undergoing total joint replacement or from human cadaveric donors within 24 hours of death. Cartilage was graded at 0 or 1 based on the Outerbridge classification system[38]. All tissue was immediately placed in phosphate buffered solution and stored at 4°C until use within 1-3 hours of harvest. All articular cartilage was kept full thickness in 10mm diameter osteochondral dowels with the cartilage on a bone base to mimic the clinical scenario required for articular cartilage transplantation.

Preliminary Experiments

Preliminary experiments were designed based on the data obtained from our previous studies on CPA vitrification properties[36], CPA permeation kinetics[34, 35], CPA toxicity[32, 39], and CPA freezing point[37]. In each vitrification protocol examined, different CPAs were added sequentially at lowering temperatures for specified periods of time. After introduction of a first CPA, additional CPAs were added in a stepwise algorithm of applying a permeating dose (for a newly introduced CPA) and a maintenance dose (for CPAs already loaded into the tissue). It is also important to note that the temperature was systematically reduced concurrent with the introduction of the last permeating CPAs and that chondroitin sulfate was not included. The combination of CPAs used included those in Table 1 which were designed based on our previously published work[32-34, 36, 37] as well as preliminary experiments. In general, the temperatures of exposure were 0°C (for the first CPA in 3-CPA solutions and for the first 2 CPAs in 4-CPA solutions), -10°C (for the second last CPA) and -15°C (for the last CPA in a 4-CPA solution). The permeation time was calculated based on the exposure temperature, type and concentration of CPA desired within the matrix (with desired concentration based on prior studies and understanding of CPA toxicities, interactions and freezing points) and the estimated thickness of the cartilage. The results from

the various experiments, including those in Table 1, resulted in the determination of our final experimental vitrification solution detailed under Experimental Protocol.

Experimental Protocol

Osteochondral dowels were obtained from discarded tissue of 10 separate individuals undergoing knee replacements. The 10mm diameter samples were removed from the best portion of the articular cartilage after visual inspection and graded 0 or 1 on the Outerbridge scale. Fresh control samples were taken from the same joint using the same criteria and immediately stained using membrane integrity stains as described below. Fresh control viability results were used to normalize the experimental sample results. Negative controls were historical controls that had been rendered negative by immersion of fresh osteochondral tissue without CPA protection into liquid nitrogen (LN_2) three times. This consistently resulted in 0% cell recovery as determined by membrane integrity stains.

Osteochondral tissue was immersed in the CPA solutions for exposure times to achieve a “minimum” concentration throughout the articular cartilage matrix for each CPA as calculated using a 2D Fick’s law diffusion model[33, 35] based on diffusion coefficients calculated in a previous study[34]. For this calculation, the specified conditions were temperature, permeation kinetic parameters, CPA concentration in the surrounding bath, and the estimated cartilage thickness. Following the CPA treatment steps, the dowels were rapidly placed into LN_2, for 30 minutes, to ensure a temperature lower than -180°C was attained within the core of the dowel. Subsequently, a rapid warming step was employed, in a water bath, at 37°C.

The details of the final vitrification protocol, referred to as “6.5M DGPE”, are given below. For this specific experiment, osteochondral tissue was exposed to 6M DMSO for 1hr 30min at 0°C to obtain a minimum 2.4375M concentration within the matrix (at the bone-cartilage junction). The tissue was

moved to the second solution containing 6M glycerol and 2.4375M DMSO for 3hr 40min at 0°C to achieve a minimum concentration within the matrix of 2.4375M DMSO and 1.625M glycerol. The tissue was moved to the third solution containing 6M PG, 2.4375M DMSO and 1.625M glycerol for 3hr at -10°C to obtain a matrix minimum concentration of 2.4375M DMSO, 1.625M glycerol, and 0.8125M PG. The tissue was moved to the fourth solution containing 6M EG, 2.4375M DMSO, 1.625M glycerol and 0.8125M PG for 1hr 20min at -15°C. After this time, the tissue was moved into the final solution of 2.4375M DMSO (18.0% w/w), 1.625M glycerol (14.1% w/w), 0.8125M PG (5.8% w/w), and 1.625M EG (9.5% w/w) (to match the existing minimum concentrations within the matrix and a total of 47.4% w/w) in a 5ml vial sealed with a cap and plunged into LN_2. All solutions contained X-VIVO 10 (VWR, Canada) and 0.1mg/ml chondroitin sulfate (Sigma-Aldrich, Canada). The tissue was held in LN_2 for a minimum of 30min (sufficient for the center of the tissue to reach -180°C – unpublished data). The vial was removed from the LN_2 and placed into a 37°C water bath until the solid solution began to soften providing an estimated warming rate of 64-122°C per minute as determined in our previous work[23, 40]. The tissue was removed from this solution with excess solution blotted off and placed into 300ml of X-VIVO 10 with 0.1mg/ml chondroitin sulfate at 4°C in a cold room in ice-water for 30min to dilute out the CPAs. It was then moved to another container of 25ml X-VIVO 10 to dilute out any residual CPAs until the sample was examined for membrane integrity as described below. During the CPA addition and removal, the samples were gently shaken on a mechanical shaker.

After completion of the primary experimental group, the exact same protocol (unless stated otherwise) was applied to other similar and related articular cartilage. This included:

1. Five 10mm diameter fresh osteochondral dowels from three deceased human donors (to document effectiveness on normal cartilage)

2. Four large fragments (approx. 12.5cm^2 each) from four total knee replacement patients with three 10mm diameter dowels from the same joints (to document ability to scale up to larger sizes)
3. One large fragment (approx. 12.5cm^2) from one deceased human donor (to document scale up on normal cartilage)
4. One 10mm dowel from a total knee replacement stored for 3 months in LN_2 (to document long term storage)

These samples were examined using membrane integrity stains as described below.

Membrane Integrity Staining

Slices of 70μm thickness were removed from the osteochondral dowels using a vibratome and placed on glass slides. Each slice was stained using membrane integrity dyes of Syto 13 (Invitrogen, Canada) and ethidium bromide (EB; Sigma) [0.1% EB with 0.45% Syto 13 mixed in PBS (v/v)] and viewed under a Leitz Dialux 22 fluorescence (440-480nm) microscope (Leica Microsystems, Richmond Hill, ON). Two representative full thickness images from each sample were recorded by digital camera (Pixera DiRactor, Pixera Corporation, Los Gatos, CA, USA) and stored on computer for later cell counting under direct vision. The cell viability in individual slices was normalized against the cell viability of fresh control slices taken from the same donor and counted in the same manner. Cell viability was calculated as:

$$\text{viability} = \frac{\text{intact cells (experimental)}}{\text{intact cells (control) + disrupted cells (control)}} \quad (1)$$

Equation (1) is a conservative method to determine cell recovery because it uses experimental intact cell count compared to fresh control total cell count to account for cell loss. In some other viability calculations (i.e., intact experimental/total experimental), cell dissolution would artificially inflate viability results by not acknowledging the previous existence of those cells. Equation (1) uses all control

cells (intact and disrupted) in the denominator, which will decrease the recorded cell viability of the experimental tissue by including those cells already disrupted by the harvesting process.

Metabolic activity by alamarBlue

Warmed samples after vitrification were washed in X-VIVO 10 supplemented with 100 units/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen) and 0.1mg/ml chondroitin sulphate for 30 minutes 4 times to remove all CPAs. The AC was shaved off the bone and divided into 1gm samples and incubated in 5ml of DMEM complete medium of high glucose (4.5mg/l) Dulbecco's modified Eagle's medium (DMEM; Himedia Laboratories Pvt-VWR, Edmonton, Canada) supplemented with 10% fetal bovine serum (FBS – PAA laboratories Inc., Etobicoke, ON), antibiotics and 100 mM HEPES (Sigma-Aldrich, Oakville Canada) buffer at 37°C for 1 day in a 6 well plate. After 24 hours incubation, the media was removed and 5ml of complete DMEM was added and the sample was further incubated for 4 days. The cartilage was washed with PBS supplemented with antibiotics and placed in 5ml of serum free medium (SFM) consisting of high glucose DMEM containing, 100 mM HEPES buffer, 100U/ml penicillin/streptomycin, 2mM L-glutamine, 1.25 mg/ml human serum albumin (Sigma-Aldrich, Oakville Canada) and 1x ITS+1 premix (Sigma-Aldrich, Oakville Canada), supplemented with 0.1 mM ascorbic acid 2-phosphate (Sigma-Aldrich, Oakville Canada), 40μg/ml L-proline (Sigma-Aldrich, Oakville Canada), 10^{-5} M dexamethasone (Sigma-Aldrich, Oakville Canada) and 10 ng/ml TGF-β1 (Medicorp Inc., Canada), mixed with 500μL alamarBlue (Invitrogen, Burlington, Canada). Fluorescence was measured in triplicate at 4hr, 8hr, 24hr, 48hr, 72hr, 96hr, 120hr, 144hr, and 168hr. A blank well that contained the same medium without cartilage was also measured in triplicate. The average of the three readings of the blank samples was subtracted from the average of the experimental samples to yield a value in relative fluorescent units (RFU). Fluorescence parameters were emission 595nm, excitation 535nm, gain 46 and integration time 40μs. Positive controls consisted of articular cartilage that did not undergo the

vitrification process nor have exposure to the CPAs. The cartilage was incubated for the same amount of time and in the same solution as the experimental samples.

Matrix production by histology/immunohistochemistry

Chondrocytes were released from the matrix after exposure to 0.15% w/v collagenase II (Cedarlane, Canada) in DMEM for 22 hours. Viable cells were plated in complete DMEM for 2 weeks with medium change every 3 days and then harvested to create 250,000 cells per culture pellet. The cells were placed in 50ml conical tubes, centrifuged for 5 minutes at 400g, re-suspended in DMEM complete, and centrifuged for 5 minutes at 400g. Excess media was removed and replaced with 250µL SFM. The tubes were incubated under 3% O_2 for 3 weeks with media changes 2 times per week. Tissues generated from the pellet cultures were fixed in 10% phosphate buffered formalin (BDH Chemicals, VWR, Edmonton, Canada), processed into paraffin wax, sectioned at 5µm and stained with 0.1% safranin O (Sigma) and counterstained with 1% fast green (Sigma) to reveal sulphated glycosaminoglycan (GAG) matrix depositions. Other sections were probed with antibodies raised against collagen type II [(II-II6B3) from Developmental Studies Hybridoma Bank at University of Iowa, USA]. Immuno-localised antigens were visualized with goat anti-mouse IgG biotinylated secondary antibody (Dako Canada Inc, Mississauga, Canada) and a streptavidin-horseradish peroxidase labeling kit with 3,3'-diaminobenzidine (Dako). Images were captured using an Omano OM159T biological trinocular microscope (Microscope Store, Virginia, USA) fitted with an Optixcam summit series 5MP digital camera and Optixcam software and assembled in Adobe Photoshop (Adobe Systems Inc. San Jose, USA).

Institutional ethical approval was obtained from the University of Alberta Ethics Review Board.

Results

Figure 1 details the results for eighteen 10mm diameter samples from ten different total knee arthroplasty donors (average age 64.3 years, range 40-78 years; 5 males and 5 females) that underwent the 6.5M DGPE vitrification protocol as described in the Materials and Methods section. The average thickness of the articular cartilage was 2.44 mm (range 1.5 – 3.5 mm). The average cell recovery was 75.4 ± 12.1% based on *in situ* Syto13/EB membrane integrity staining using our conservative calculation as described by Equation 1 in the Materials and Methods section. Figure 2 presents a typical photographic image of the cell recovery in a full thickness slice after vitrification.

After the primary experiment results were obtained as detailed in Figure 1, the protocol was applied to other groups of articular cartilage to check for cartilage source and tissue size independence and cell recovery, based on Syto13/EB membrane integrity, was found to be similar for all groups. Intact cell recovery from 10mm diameter dowels of normal articular cartilage from three *deceased human donors* was 76.1%, 91.4%, 84.1% (three separate dowels) from a 21 year old male, 81.7% from a 38 year old male and 71.8% from a 57 year old male (total n=5; average = 81.0 ± 7.5%). The cell recovery from *four large fragments* (approximately 12.5cm^2) was 76.9 ± 6.2% (n=4, age range 52 – 82 years) while the corresponding 10mm diameter dowels from the same donors had a recovery of 82.8 ± 10.3% (n=3, age range 52-82 years). There was no significant difference in cell recovery between the larger fragment versus the 10mm diameter dowel from the same patients (P = 0.38). The large fragment cell recovery from the one deceased human donor (57 year old male) was 86%. The cell recovery from a dowel stored in LN_2 for 3 months (21 year old male) was 76.1%.

To confirm the functionality of the cells after vitrification, metabolic function was determined using a mitochondrial assay (alamarBlue) in two separate samples. Figure 3 shows the relative fluorescence of the vitrified dowels compared to two control dowels. By 6 days incubation in alamarBlue, the vitrified dowels demonstrated over 100% activity compared to the control dowels at the same time point. There

was a gradual increase in mitochondrial activity noted during the 7 days studied in the vitrified samples while the fresh controls reached their maximum after 24-48 hours. The maximal fluorescence after vitrification occurred at 168 hours and was approximately 95% of the maximal fluorescence exhibited by the fresh controls after 48 hours.

Further evidence of chondrocyte function post vitrification was demonstrated by pellet culture under hypoxic conditions followed by histology/immunohistochemistry for articular cartilage specific structural elements of sulfated glycosaminoglycans and collagen II (Figure 4). The vitrified pellets stained positive for both safranin O and collagen II with similar intensity and distribution as the fresh control pellets indicating that the vitrified cells were able to produce these articular cartilage specific components.

Discussion

Osteochondal allografting has the potential to be useful to treat large joint defects that are otherwise not amenable to surgical treatment, especially in younger and more active patients. Tissue access for partial[41] or whole[42] joint replacement has been limited due to the inability to store this tissue in good condition for long periods of time. Attempts to hypothermically store articular cartilage at 4°C have had limited success because deterioration begins after 7-14 days[12].

The current report documents that full thickness, intact human articular cartilage on a transplantable bone base can be cryopreserved with good to excellent cell recovery. We have demonstrated that this can be done in smaller 10mm osteochondral dowels and that it potentially may be scaled up to larger fragments without loss of cell recovery. Furthermore, we have demonstrated that once the cartilage has been vitrified, it can be stored for at least three months without significant loss of cell recovery. Our results are reproducible on cartilage obtained from knee joints undergoing joint replacement as well as from deceased human donors. We have proven the cell viability using membrane integrity,

metabolic/mitochondrial function and by documentation of the ability of these cells to grow in culture and produce cartilage specific structural elements such as sulfated glycosaminoglycans and collagen II.

For articular cartilage, initial standard cryopreservation techniques using controlled ice formation have had minimal success[21, 43] with the exception of thin sheep articular cartilage[20]. After extensive investigation, it became clear that this technique would not be successful in intact human articular cartilage[21] and investigators moved to vitrification. The advantage of this technique is that ice formation is avoided. Ice formation is lethal to cells[44] and can damage the matrix[23, 45]. The disadvantage of vitrification is that high concentrations of CPA s are required which are toxic to cells and a rapid cooling rate is required which is difficult to achieve in biologic tissues. Vitrification is used successfully in some cells[15, 16, 18] but application to tissues and organs such as the kidney[46] have proven much more difficult. Fahy did much of the pioneering work in this area and proved that vitrification of large biologic tissues is possible[27] and that combining CPAs might be more effective than using a single CPA. Brockbank achieved some success with combining CPAs and recently was able to use a combination solution to cryopreserve pig cartilage that had been removed from its bone base[30]. Removal of the cartilage from its bone base will enhance permeation of CPAs into AC because it effectively doubles the surface area for permeation. Problematically, cartilage removed from bone cannot be used clinically because there is no way to attach the vitrified cartilage. Furthermore, removing the cartilage from the bone will change its physical properties because the bone anchor is essential to cartilage function as noted by the progression of cartilage deterioration when it is delaminated in the clinical scenario. The process described herein can be applied to other tissues/organs and potentially revolutionize transplantation surgery. Our studies to address each of CPA vitrifiability, CPA permeation and CPA toxicity required the collaborative use of mathematical modeling and statistical methodology, and the results obtained allowed us to develop the current vitrification protocol. With a limited amount of experiments compared to the infinite number of combinations available, we were able to determine that

the current D-G-P-E concentrations and application in the stated order at the specified temperatures and quantified permeation times was able to successfully vitrify intact human articular cartilage. In previous work at comparable cooling and warming rates, we arrived at predictive equations for vitrifiability (on the basis of visual observation of ice) of CPA solutions including DMSO, glycerol, EG and PG[36]. According to this work, our final solution in the absence of AC is predicted to be vitrifiable and likely to have only partial devitrification or no devitrification upon warming. In these experiments with the articular cartilage present, no ice was observed. One important element of our current protocol includes the use of a combination of CPAs. It has been shown that, in general, combinations of CPAs at a specific total molality are less toxic than single CPAs at the same molality[32, 47, 48]. Another important element is the addition of the CPAs at sequentially lowering temperatures. CPAs are less toxic at lower temperatures[49]. Adding two of the least toxic CPAs at the highest temperature allowed us to add the more toxic CPAs at lower temperatures without the risk of ice formation; thereby decreasing the overall toxicity of the process. The order of addition may be important as noted by the lower cell recovery when the order of CPAs was changed (Table 1). X-VIVO was used based on success in hypothermic studies including one on articular cartilage[50]. Finally, the addition of chondroitin sulfate significantly improved the cell recovery. As can be seen from the preliminary data (Table 1), the same vitrification solution and addition protocol, in the absence of chondroitin sulfate, had an average of 52.4% cell recovery. Once the chondroitin sulfate was added, this increased to 75.4%. The mechanism of action for chondroitin sulfate is not clear but it has been beneficial in other preservation solutions for tissues such as corneas[51, 52] and veins[53]. One final essential step to mitigate the toxic effects of high concentrations of CPAs was to expose the tissue to the CPAs for the shortest period of time possible. To accomplish this, we utilized mathematical calculations of the desired minimum concentration (based on freezing points) and of the specific duration of exposure to achieve that minimum concentration of a specific CPA throughout the matrix. For example, we computed the time required for the cartilage to be exposed to 6M DMSO to

achieve a minimum concentration of 2.4375M DMSO throughout the cartilage matrix. There was some variability in the cartilage thickness; therefore there could be some degree of variability in the CPA concentration within the matrix. To compensate for this, the solution for the next step of the process would contain 2.4375M DMSO so that the concentration could equilibrate throughout the tissue. This was repeated for each step of the process. If the exposure time is too short, insufficient concentration of the specific CPA would occur resulting in ineffective vitrification, ice formation and excessive cell death. Too long of an exposure time would result in excess toxicity with increased cell death.

Evaluating the results of cartilage vitrification and preservation in general has been difficult. Freeze injury to chondrocytes is primarily concentrated on cellular membranes[54, 55]; therefore, membrane integrity stains have been chosen as a reliable indicator of cell recovery in cryopreserved tissues[20, 56]. Alternatively, it has been suggested that membrane integrity stains are not reliable based on a study performed with Calcein AM[57]. Calcein AM is a cytoplasmic stain that undergoes chemical alteration due to cytoplasmic enzymes; therefore, this stain can indicate cell recovery even without cellular nuclei resulting in an overestimation of cell recovery. We agree that membrane integrity stains demonstrate the best possible cell recovery and that cytoplasmic stains can overestimate cell recovery. For these reasons we have used Syto 13 for many years. Syto 13 is a nuclear membrane binding stain. Therefore, all of the cells that we identify as staining positive for Syto 13 must have an intact nucleus. Furthermore, both Syto 13 and EB are nuclear membrane binding stains therefore one will not overpower the other based on volume alone as can occur when comparing a cytoplasmic stain (i.e. Calcein AM) with a nuclear stain (i.e. EB). We have used the strictest criteria for determining the percentage of cells recovered. We used only those cells staining green (intact) after vitrification divided by the *total* number of cells (green and red) from the control. This means that any cells lost during the harvest, transportation and set up time would be attributed to the cryopreservation process. A more liberal technique often used is to compare the green cells after vitrification to the number of green cells in the control tissue. This eliminates all of those

cells that died during the processing before the vitrification process. This may more accurately reflect the success of the vitrification process itself. Using this calculation, our result was 81.1%.

Membrane integrity stains document that cells are intact but cannot indicate the cellular functionality. Unfortunately, metabolic stains can result in falsely high cell recovery estimations especially after hypothermic storage[58] possibly due to chondrocyte stress or persistently active enzymes despite cell breakdown. To document viability in this study, we performed two tests. alamarBlue was used because it is an indicator of mitochondrial respiratory function. It was clear that the experimental cells required a few days to recovery after the vitrification process. Once recovered, these cells demonstrated 95% function relative to the maximal response of the fresh control tissues. The alamarBlue assay clearly demonstrated that a good portion of the cells are surviving the vitrification process and are metabolically active.

The second and more comprehensive and specific confirmatory test was the demonstration of the production of articular cartilage specific structural elements of sulfated glycosaminoglycans and collagen II. This test confirmed that the chondrocytes in the vitrified articular cartilage are functional as healthy chondrocytes and have the capacity to contribute to matrix turnover after transplantation.

This study has its limitations. Further optimization may be achieved. We did not have complete control over the specimen from the operating room and deceased donors. Improving the harvesting technique such as minimizing the exposure of the cartilage to air, placing the cartilage immediately into an optimized holding solution containing chondroitin sulfate, and rapid vitrification processing will further improve cell recovery. Once this process nears clinical practice, it will be possible to institute these measures. The length of time that tissue can be held in LN_2 has not been determined. Once the tissue has achieved a temperature lower than -130°C, no appreciable biological or chemical reactions occur. To

reach this temperature takes less than 20 minutes in our conditions. Therefore we believe that 3 months is sufficient time to verify the potential of this protocol to store tissues indefinitely.

Conclusions

The results of this study are important for two reasons. Firstly, this study documented that intact human articular cartilage can be cryopreserved with excellent cell recovery and cell functionality. This research will form the basis for the development of an osteochondral tissue bank with vitrified human articular cartilage that can provide healthy tissues for joint transplantation. An effective transplantation program can decrease the incidence of osteoarthritis thereby decreasing the massive socioeconomic burden on our society. Secondly, the interdisciplinary process described in this study of directed biologic experiments combined with mathematical modeling and statistical methodology may possibly be applied directly to other tissues and organs with a significant effect on the availability of those tissues and organs for transplantation.

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Conflict of Interest: NMJ, JAWE, LEM, FF, GKL, BM, and AA have submitted a patent related to this research. This patent does not currently have any financial value.

Figure Captions

Figure 1. Color-coded graph demonstrating the intact cell recovery based on *in situ* Syto13/EB membrane integrity stains of 18 dowels from 10 different human total knee arthroplasty cartilage donors. Each color and number represents a different donor. The number of samples from each donor was dependent on the quality of the cartilage obtained. The chondrocyte recovery is normalized to control samples from the same donor according to Equation (1) stated in the Materials and Methods section.

Figure 2. Four digital images stitched together spanning the full thickness (2-3mm) of human articular cartilage. (a) fresh control and (b) experimental sample from the same patient after vitrification using a solution of 2.43M DMSO (w/w), 1.65 glycerol (w/w), 0.81M PG (w/w), 1.63M EG (w/w) with 0.1mg/ml chondroitin sulfate with each CPA added at progressively lower temperatures and subsequent warming. Subsequent staining with Syto 13 and ethidium bromide highlights intact (green) and disrupted (red) cells. Manual counting of cells recorded 77% intact cell recovery in this human tissue after the vitrification procedure.

Figure 3. alamarBlue fluorescence in the vitrified sample normalized to the fresh control sample. Note that the vitrified sample achieved greater than 100% of the control sample at 144hrs. The maximal fluorescence of the vitrified sample at 168hr was approximately 95% of the maximal fluorescence of the control sample (that occurred at 48hrs). It took approximately 12 days for the chondrocytes to recover from the vitrification process – 5 days in DMEM and then 7 days in serum free media and alamarBlue. The results presented compare the average of two separate vitrified samples from two different total knee arthroplasty donors to the average of two fresh controls from the same donors.

Figure 4. Histological and immunohistochemical images after 3 weeks of pellet culture for fresh controls (a, c) and vitrified samples (b, d). Figures 4a (fresh control) and 4b (vitrified sample) demonstrate safranin O distribution (red/pink) for sulfated glycosaminoglycans throughout the pellet of similar intensity. Figures 4c (fresh control) and 4d (vitrified sample) demonstrate collagen II distribution of

similar intensity in both samples. For comparison, Figure 4e is a collagen II negative control (fresh tissue) without the collagen II primary antibody. Magnification is 10X objective with 0.5X magnification on the digital camera. Histology and immunohistochemistry were repeated with two separate samples (results from the second sample not shown). Scale bar = 100μm.

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Table

Table 1. Selected preliminary experimental combinations and corresponding results.

Combination	CPA ratio	Total concentration	Average recovery
D-G-P-E	3-2-1-2	6.5M	52.4%
D-G-P-E	3-2-1-2	8M	50.1%
G-D-F	1-1-1	6.5M	50.8%
G-D-F	1-1-1	8M	37.5%
G-D-F-E	1-1-1-1	6.5M	51.0%
G-D-F-E	1-1-1-1	8M	43.5%
G-E-F-D-P-Me-Et	4-4-1-1-1-1-1	6.5M	48.0%
G-E-F-D-P-Me-Et	4-4-1-1-1-1-1	8M	31.8%
E-G-D-P	2-2-3-1	6.5M	40.3%
E-G-D-P with CS	2-2-3-1	6.5M	53.0%

D = DMSO, G = glycerol, P = propylene glycol, E = ethylene glycol, F = formamide, Me = methanol, Et = ethanol, CS = chondroitin sulfate. N = 3 separate samples for each combination (N = 1 with 2 replicates for E-G-D-P).

Figure 1
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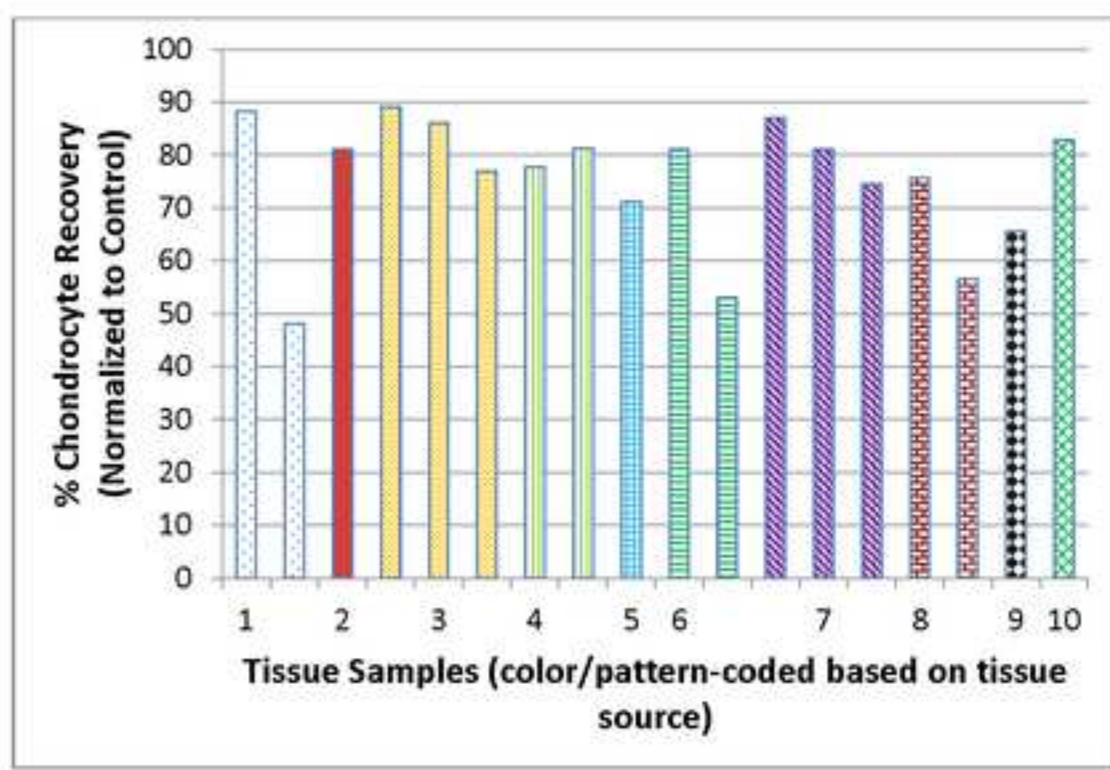

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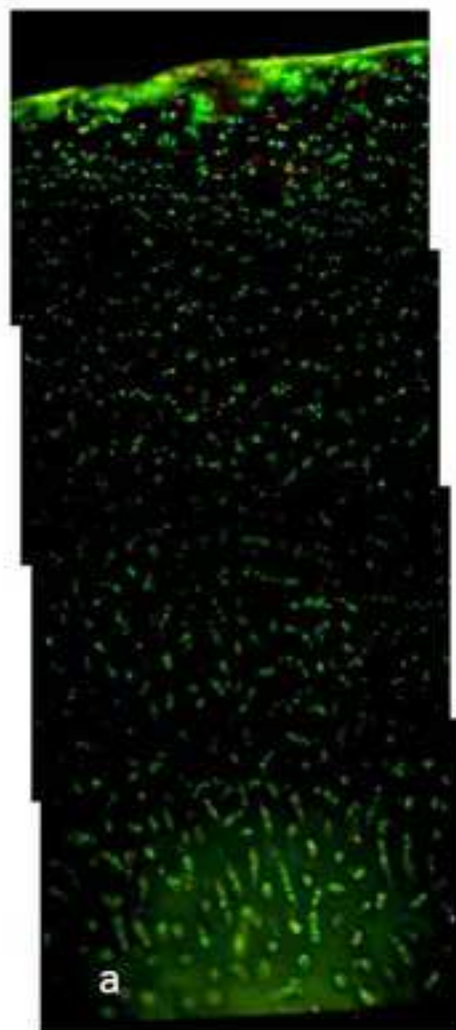

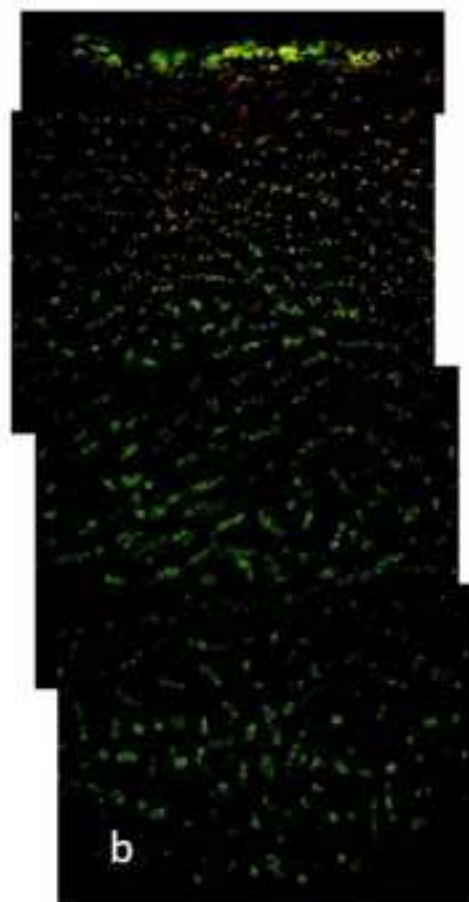

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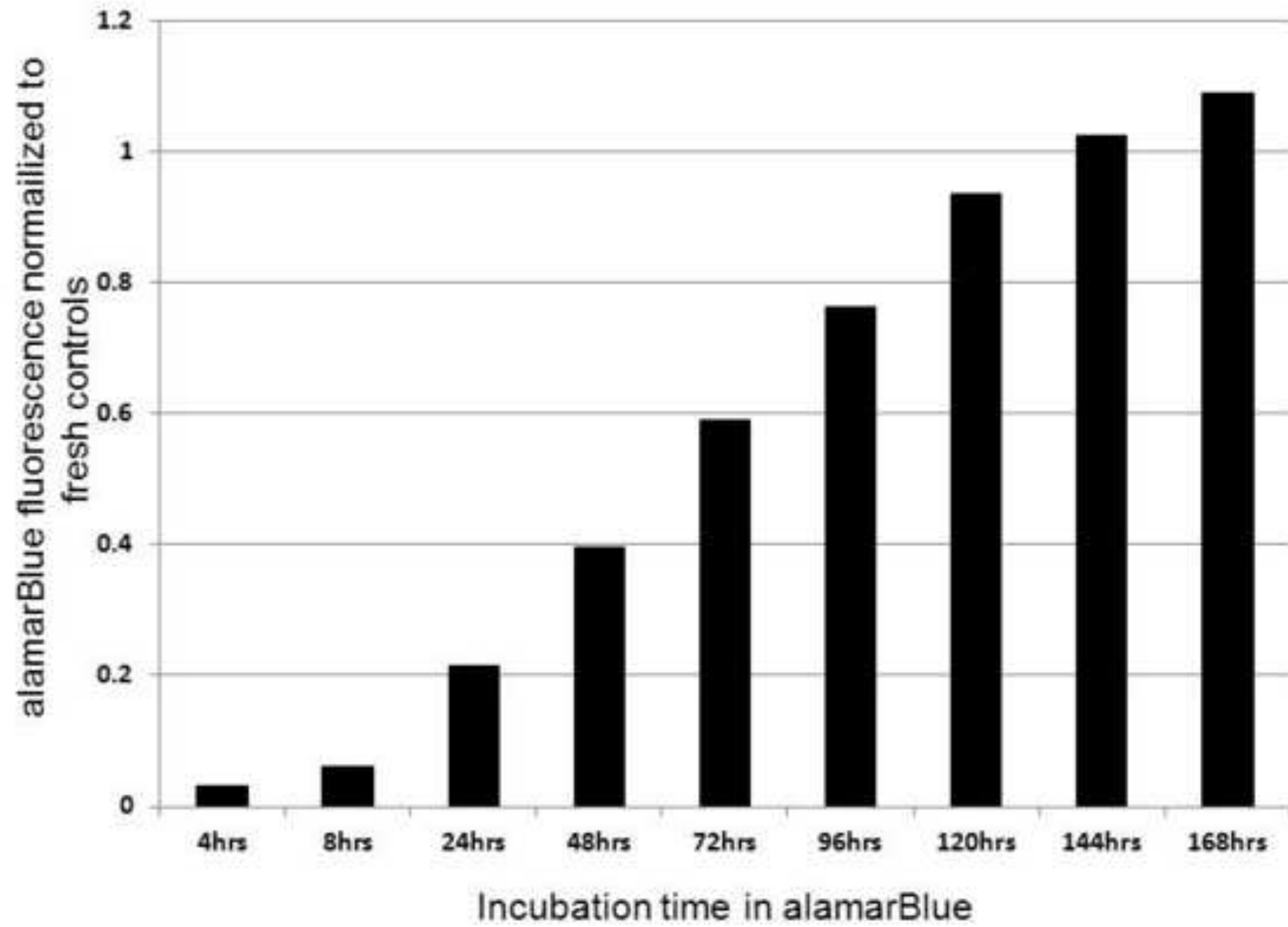

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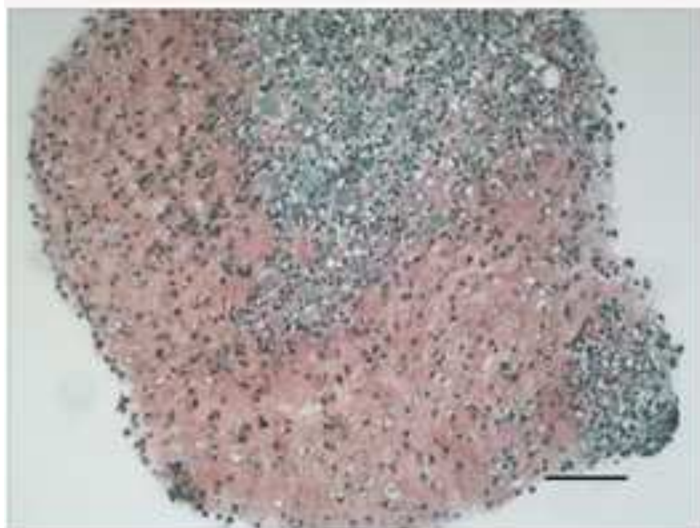
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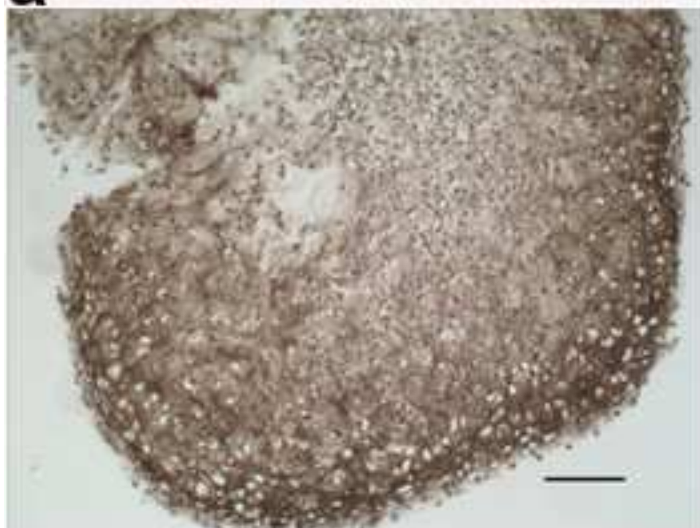
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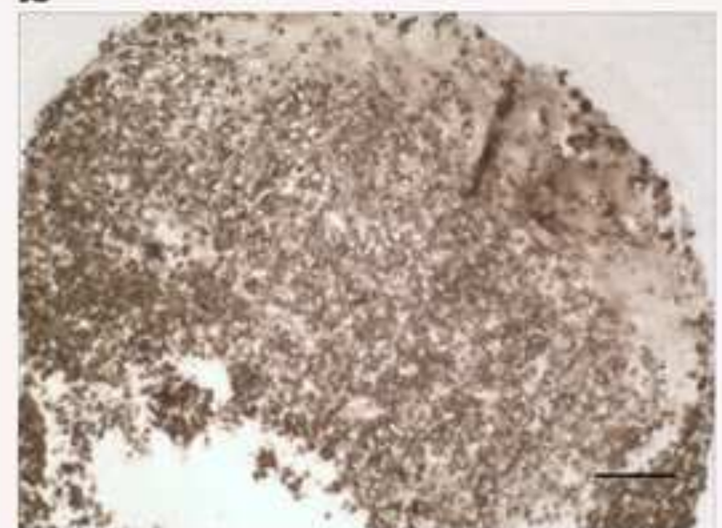
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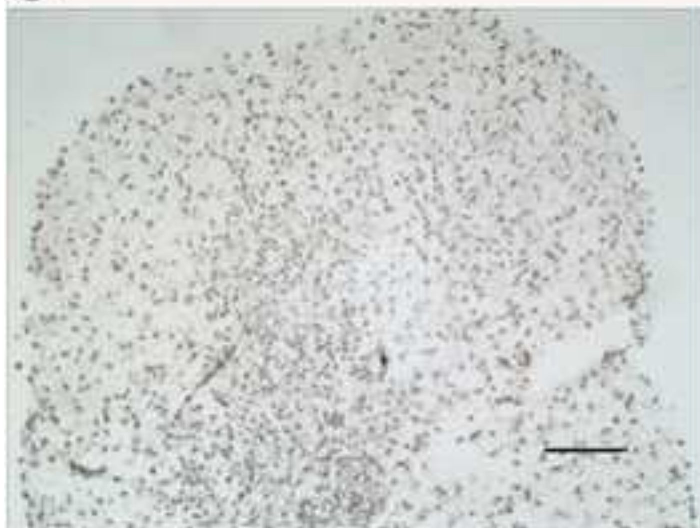
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