

# Vitrification of Intact Porcine Femoral Condyle Allografts Using an Optimized Approach

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## Abstract

**Objective.** Successful preservation of articular cartilage will increase the availability of osteochondral allografts to treat articular cartilage defects. We compared the effects of 2 methods for storing cartilage tissues using 10-mm diameter osteochondral dowels or femoral condyles at  $-196^{\circ}\text{C}$ : (a) storage with a surrounding vitrification solution versus (b) storage without a surrounding vitrification solution. We investigated the effects of 2 additives (chondroitin sulfate and ascorbic acid) for vitrification of articular cartilage. **Design.** Healthy porcine stifle joints ( $n = 11$ ) from sexually mature pigs were collected from a slaughterhouse within 6 hours after slaughtering. Dimethyl sulfoxide, ethylene glycol, and propylene glycol were permeated into porcine articular cartilage using an optimized 7-hour 3-step cryoprotectant permeation protocol. Chondrocyte viability was assessed by a cell membrane integrity stain and chondrocyte metabolic function was assessed by alamarBlue assay. Femoral condyles after vitrification were assessed by gross morphology for cartilage fractures. **Results.** There were no differences in the chondrocyte viability ( $\sim 70\%$ ) of 10-mm osteochondral dowels after vitrification with or without the surrounding vitrification solution. Chondrocyte viability in porcine femoral condyles was significantly higher after vitrification without the surrounding vitrification solution ( $\sim 70\%$ ) compared to those with the surrounding vitrification solution (8% to 36%). Moreover, articular cartilage fractures were not seen in femoral condyles vitrified without surrounding vitrification solution compared to fractures seen in condyles with surrounding vitrification solution. **Conclusions.** Vitrification of femoral condyle allografts can be achieved by our optimized approach. Removing the surrounding vitrification solution is advantageous for vitrification outcomes of large size osteochondral allografts.

## Keywords

articular cartilage, osteochondral allografts, femoral condyle, vitrification, tissue banking

## Introduction

Articular cartilage defects are a common injury treated in orthopedic clinics around the world.<sup>1,2</sup> Cartilage defects can develop into osteoarthritis without proper intervention or treatment, especially in young and active adults who often suffer from acute trauma to the knee.<sup>2,3</sup> Osteochondral allografting has proven to be an effective surgical procedure to treat articular cartilage defects.<sup>4-6</sup> Depending on the size of the articular cartilage defect, the procedure requires grafts that range from small to large pieces of osteochondral tissue. Fresh articular cartilage grafts are the gold standard used for cartilage repair in orthopedic surgery. Fresh articular cartilage grafts can be stored up to 28 days but may not be delivered to patients in the operating room in time due to the long time frame of surgical preparation. Each graft requires regulatory clearance for infectious disease testing, graft size matching, patient preparation, and arrangement of

a surgical suite.<sup>7-10</sup> The short storage period of fresh articular cartilage grafts and the absence of chondrocyte survival and matrix distortion in frozen grafts<sup>5,9</sup> makes the long-term preservation of large articular cartilage grafts very important. Successful cryopreservation of articular cartilage can

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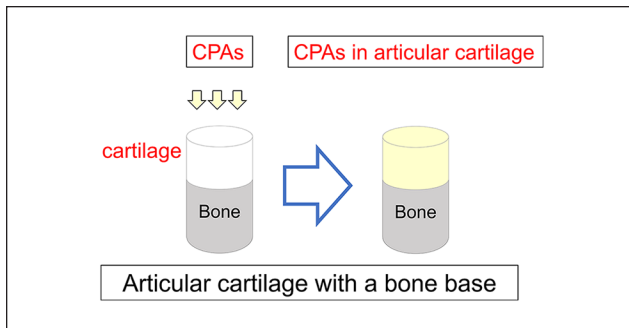
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**Figure 1.** Cryoprotectant permeation into the articular cartilage graft with a bone base before plunging into liquid nitrogen for vitrification.

increase the availability of articular cartilage allografts as an alternative transplantation source to treat large articular cartilage defects in clinical practice.

Vitrification is an “ice-free” preserving method developed for long-term storage of cells and tissues in a “glassy” solid state at extremely low temperatures (e.g.,  $-196^{\circ}\text{C}$ ). Cryopreservation by vitrification has been reported to preserve small articular cartilage grafts such as rabbit,<sup>11</sup> porcine,<sup>12,13</sup> and human<sup>14</sup> osteochondral grafts with a size from 3-mm to 10-mm diameter. However, vitrification of full knee condyles has not yet been reported in the literature, and this is important because of the potential for surgical repair of large articular cartilage defects.<sup>14,15</sup> In current practice, a sufficient amount of cryoprotectant (CPA) needs to be permeated into the articular cartilage for successful vitrification (see **Fig. 1**).<sup>14</sup> CPA permeation is the essential step to avoid ice formation in the articular cartilage matrix during the cooling/warming processes of vitrification.<sup>12,14,16,17</sup> The development of CPA permeation protocols can be optimized using mathematical modelling. Our group developed an optimized 7-hour stepwise CPA permeation protocol for articular cartilage with 2-mm thickness,<sup>18</sup> and the experimental results confirmed the efficiency of this protocol for 10-mm diameter porcine osteochondral dowels with a promising chondrocyte survival of approximately 75%.<sup>13</sup> Since our CPA permeation approach leads to a vitrifiable concentration of CPAs throughout the cartilage tissue and this has been successful in smaller osteochondral tissue fragments,<sup>18</sup> it is essential to document its effectiveness on a larger scale such as full femoral condyles. Unfortunately, vitrification of full femoral condyle articular cartilage is challenging due to the large volume of tissue. Tissue cracking caused by inhomogeneous thermal expansion of the glassy CPAs around the sample is an unsolved problem. Removing the surrounding CPAs from the tissue before storage in liquid nitrogen may be an appropriate approach to mitigate the cracking effect for the vitrification of large tissues. Using additives to protect cells from exposure to

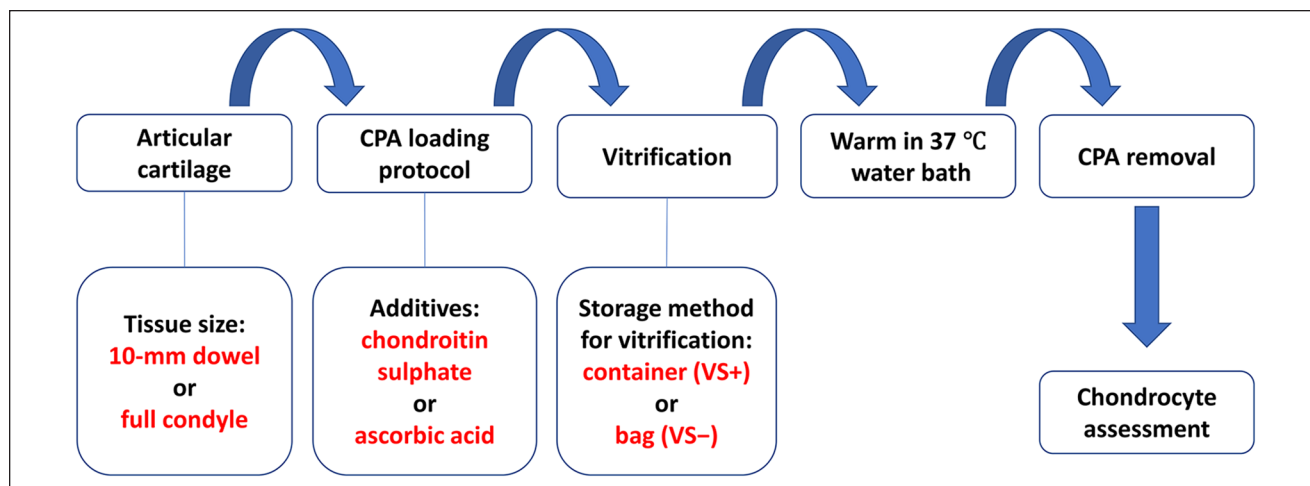
CPAs is another approach to improve cryopreservation protocols.<sup>19</sup> Additives such as chondroitin sulfate, ascorbic acid, or glucosamine have been shown to improve porcine chondrocyte survival after exposure to a high molarity CPA cocktail solution.<sup>20</sup>

Therefore, we present the vitrification of intact porcine femoral condyles using an optimized approach based on an established 7-hour protocol. Our aim is to develop a successful protocol for long-term storage of intact femoral allografts via vitrification for clinical repair of articular cartilage defects. Our objectives with this experiment were to compare vitrification results of 10-mm diameter osteochondral dowels and femoral condyles when stored with/without surrounding vitrification solution and the effects of chondroitin sulfate and ascorbic acid on cell viability after vitrification. We hypothesized that intact porcine femoral articular cartilage can be successfully vitrified once sufficient concentrations of CPA have permeated into the cartilage matrix and chondrocytes, even if the surrounding vitrification solution is removed before vitrification. We compared the effects of 2 methods for storing cartilage tissues using 10-mm diameter osteochondral dowels or femoral condyles at  $-196^{\circ}\text{C}$ : (a) storage with a surrounding vitrification solution versus (b) storage without a surrounding vitrification solution. We investigated the effects of 2 additives (chondroitin sulfate and ascorbic acid) for vitrification of articular cartilage.

## Materials and Methods

### Preparation of Articular Cartilage

Healthy porcine stifle joints ( $n = 11$ ) from sexually mature pigs (age approximately 54 weeks) were collected from a slaughterhouse within 6 hours after slaughtering for commercial consumption. No animals were specially sacrificed for this research project. The use of animal tissue for research was approved by the Research Ethics Office at the University of Alberta. Porcine joints were immersed in phosphate-buffered saline (PBS) and transported to the university laboratory in a cooler bag. On arrival in the laboratory, the joints were dissected and the femoral condyles were isolated from the tibia bone in a fume hood designated for animal sample processing, followed by 15-minute cleaning in 100 mL PBS + antibiotics (100 units/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 0.25  $\mu\text{g}/\text{mL}$  amphotericin B; Gibco) under a sterile biosafety cabin. After the cleaning, the femoral condyles were immersed in sterile DMEM complete medium (Dulbecco’s Modified Eagle Medium Nutrient Mixture F12 [DMEM-F12; Gibco] supplemented with 10% calf bovine serum, 1 mM sodium pyruvate, 100 units/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin and 0.25  $\mu\text{g}/\text{mL}$  amphotericin B [Gibco]) and kept in the fridge at  $4^{\circ}\text{C}$  until the vitrification experiments. When osteochondral



**Figure 2.** Experimental variables (in red text) for vitrification of articular cartilage graft.

dowels were required, they were cut from the tissue with a sharp cutting device with a 10-mm diameter opening.

### Experimental Variables

Experimental variables for the vitrification experiments are shown in red text in **Figure 2**. We evaluated 2 storage methods for articular cartilage vitrification (with surrounding solution in a container, VS+; or without surrounding solution in a bag, VS-) using both 10-mm diameter osteochondral dowels (cartilage on a 10-mm thick bone base) and full femoral condyles (approximate length  $\times$  width  $\times$  height: 50  $\times$  30  $\times$  20 mm<sup>3</sup>). In addition, we evaluated the inclusion of additives (chondroitin sulfate, CS; or ascorbic acid, AA) to compare their effects on chondrocyte survival after vitrification.

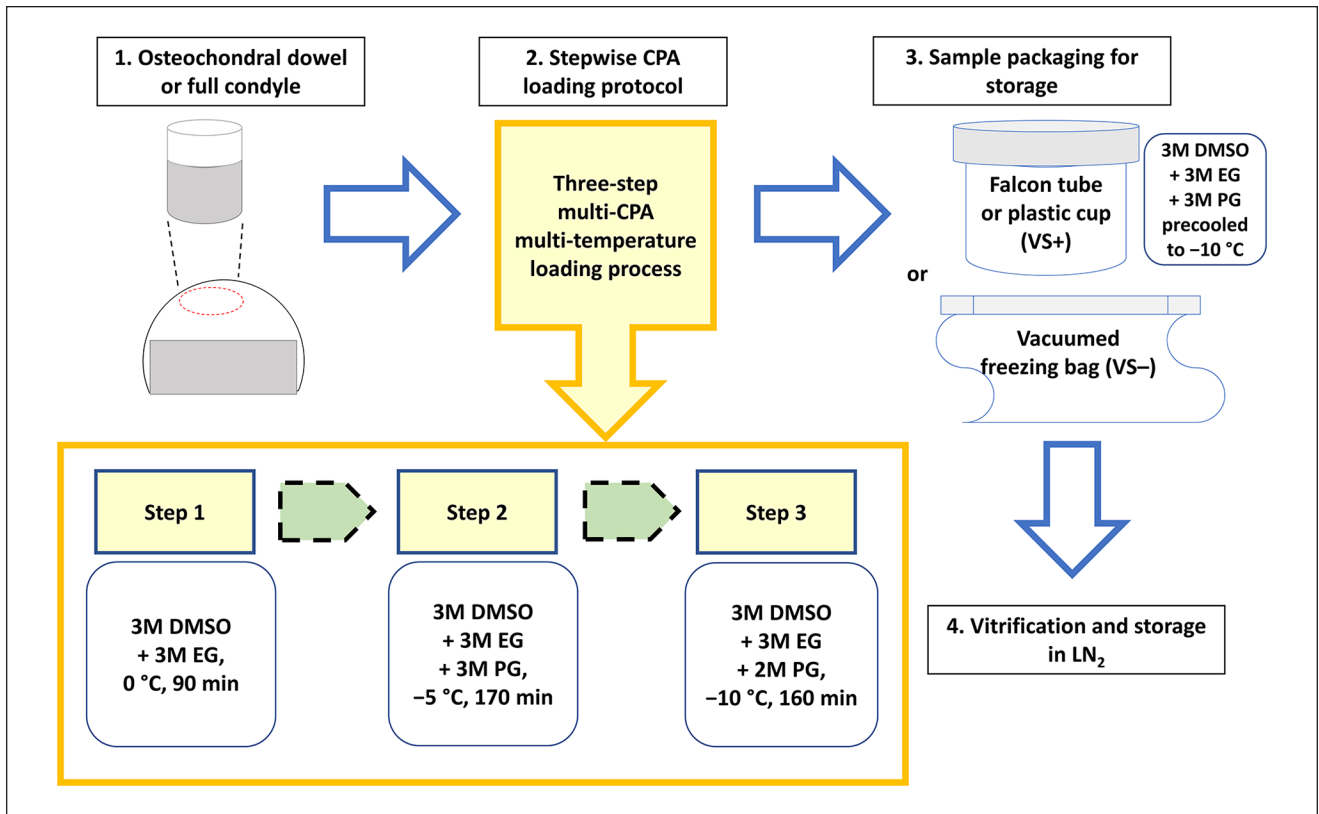
### Vitrification Flowchart

During the vitrification experiments, samples of either 10-mm diameter osteochondral dowels or full femoral condyles were exposed to the multi-CPA loading solution (by immersion in 50 mL for dowels or 200 mL for full femoral condyles) prepared with DMEM-F12 medium following the 7-hour 3-step loading protocol developed previously<sup>13,18</sup>; see **Figure 3** for the CPA concentrations, durations, and temperatures of each step indicated in the orange box. Once the CPA loading procedure was completed, the cartilage samples were transferred to the containers (15 mL Falcon tube with 5 mL vitrification solution for the dowels, 250 mL plastic cup with 100 mL vitrification solution for the femoral condyles, VS+ in **Fig. 3**, see **Fig. 4A**), or to the freezing bags (one sample per bag, for both dowels and femoral condyles, VS- in **Fig. 3**, see **Fig. 4B**) which were vacuumed to remove the air and sealed quickly with a packaging machine. Solution 2 (3 M DMSO + 3 M EG + 3 M PG) prepared with DMEM-F12 was precooled to  $-10^{\circ}\text{C}$

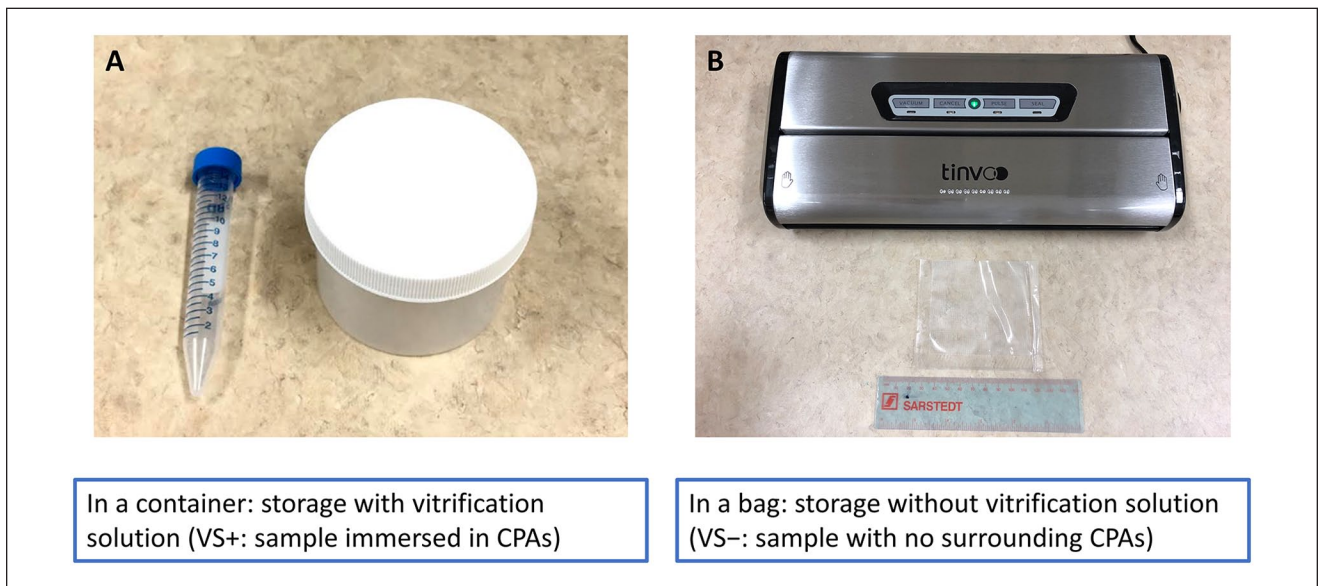
and used as the vitrification solution for packaging, VS+. All samples (prelabelled with ID number and experiment date) were then plunged into the LN<sub>2</sub> Dewar for storage.

### Assessment of Articular Cartilage

**Chondrocyte Viability via Cell Membrane Integrity Stain.** Chondrocyte viability was quantified via cell membrane integrity using fluorescent microscopy similar to our previous work.<sup>13</sup> After tissue warming and CPA removal, a vibratome (vibratome-1000 plus, the Vibratome Company, St. Louis, MO) was used for sectioning cartilage slices. The vibratome basin was filled with 500 mL 1 $\times$  PBS (4°C) to avoid cartilage dehydration during sectioning. The osteochondral dowel was placed in a metal sample holder and cartilage slices with a thickness of 100  $\mu\text{m}$  were sectioned in a transverse plane, then transferred to one labelled well of a 24-well plate filled with 2.0 mL X-Vivo 10 (Lonza) and kept on crushed ice with distilled water before sample staining. For imaging, a mixture of 2 fluorescent dyes: 6.25  $\mu\text{M}$  Syto 13 (Molecular Probes) and 9  $\mu\text{M}$  propidium iodide (PI; Sigma) were used to label membrane-intact (live cell, green color) and membrane-damaged (dead cell, red color) chondrocytes. Cartilage slices were placed on labelled microscope slides and excess X-Vivo on the slices was removed with Kimwipe. Each slice was overlaid with approximately 50  $\mu\text{L}$  stain mixture and covered with a coverslip. Cartilage slices were incubated in the dark for 10 to 15 minutes to allow dye permeation into chondrocytes. Cartilage slices were imaged using a Nikon digital camera (model: DS-Fi2) under a Nikon inverted fluorescent microscope (model: ECLIPSE Ti-5). Dual filters with the following spectra peak maxima: excitation/emission: 488 nm/503 nm and 535 nm/617 nm were used to image all the slices. The cell viability for each cartilage slice was determined by counting the numbers of the green-stained (viable) cells and red-stained (nonviable)



**Figure 3.** Flowchart for vitrification of articular cartilage graft using a 3-step cryoprotectant loading protocol.



**Figure 4.** The 2 packaging methods for vitrification of articular cartilage graft. **(A)** A 15-mL Falcon tube for the osteochondral dowel and a 250-mL plastic container with a white lid for the condyle to be vitrified with surrounding CPAs. **(B)** A freezing bag and the vacuumed machine for the osteochondral dowel and the condyle to be vitrified without surrounding CPAs.

cells, using custom made software Viability 3.2 (Locksley McGann, University of Alberta).<sup>21</sup> An inclusion criteria of positive control cell viability from fresh cartilage slices of

greater than 85% was used to screen healthy cartilage for the study. Normalized cell viability of the experimental samples was determined according to the following formula:

Normalized cell viability =

$$\frac{\left( \frac{\text{\# of green cells after vitrification}}{\text{\# of green cells + \# of read cells after vitrification}} \right)}{\left( \frac{\text{\# of green cells before CPA loading}}{\text{\# of green cells + \# of read cells before CPA loading}} \right)} \times 100\% \quad (1)$$

### Chondrocyte Metabolic Activity Assessed with AlamarBlue

Chondrocyte metabolic activity was determined by alamarBlue as documented in our previous work.<sup>13</sup> AlamarBlue is a fluorescence indicator based on the reduction reaction of metabolically active cells to convert the blue-colored resazurin (nonfluorescent) into red-colored resorufin (highly fluorescent). Briefly, after tissue warming and CPA removal, cartilage from each experimental group was removed from the osteochondral dowel bone base and weighed and washed in 5 mL sterile PBS supplemented with antibiotics for 15 minutes. Positive controls consisted of articular cartilage that was neither exposed to CPAs nor vitrified; negative controls were articular cartilage plunged into liquid nitrogen without CPAs. Cartilage samples were then incubated in an alamarBlue (Invitrogen, Burlington, Canada) assay solution containing 5 mL X-VIVO 10 (a serum-free medium; Lonza) supplemented with 0.1 mM ascorbic acid, 100 nM dexamethasone, and 10 ng/mL transforming growth factor beta 1, and mixed with 500  $\mu$ L alamarBlue in a 6-well plate and incubated at 37°C for fluorescence readings every 24 hours for 4 days using Cytofluor 2.0 software. The fluorescence parameters were set to emission wavelengths of 580/50 nm, excitation wavelengths of 485/20 nm, and a gain of 45. Fluorescence was measured for each sample per experimental group at 24 hours, 48 hours, 72 hours, and 96 hours. Readings of blank samples (alamarBlue assay solution without cartilage samples) were subtracted from readings of the experimental samples to yield a value in relative fluorescent units (RFU) divided by weight in grams of articular cartilage. The RFU readings of chondrocytes after vitrification were normalized to the fresh positive controls and presented as a percentage in the figures.

### Cooling and Warming Temperature Profile of Full Femoral Condyles

To compare the cooling and warming rates of articular cartilage stored with different methods, a dual thermometer with 2 thermocouple detectors was used to determine the temperature as a function of time of full femoral condyles ( $N = 3$  condyles per group, in a container or in a bag) as they were cooled from  $-10^{\circ}\text{C}$  to  $-196^{\circ}\text{C}$  then warmed to  $37^{\circ}\text{C}$ . Two 2-mm-deep holes were drilled on the weight bearing area of the condyle cartilage surface to place the thermal detectors for temperature measurement. The temperatures of articular cartilage at different time

points (0 minutes, 1 minute, 3 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes) were recorded and plotted to observe the cooling and warming rates of articular cartilage.

### Gross Morphology of Full Femoral Condyles after Vitrification

To compare the effects of packaging methods on the morphology of femoral condyles after vitrification, cartilage fractures on condyle surface were identified by gross imaging. After tissue warming and CPA removal from the vitrified femoral condyles, the condyles were placed on a paper towel and imaged using a digital camera (Canon PowerShot ELPH 180).

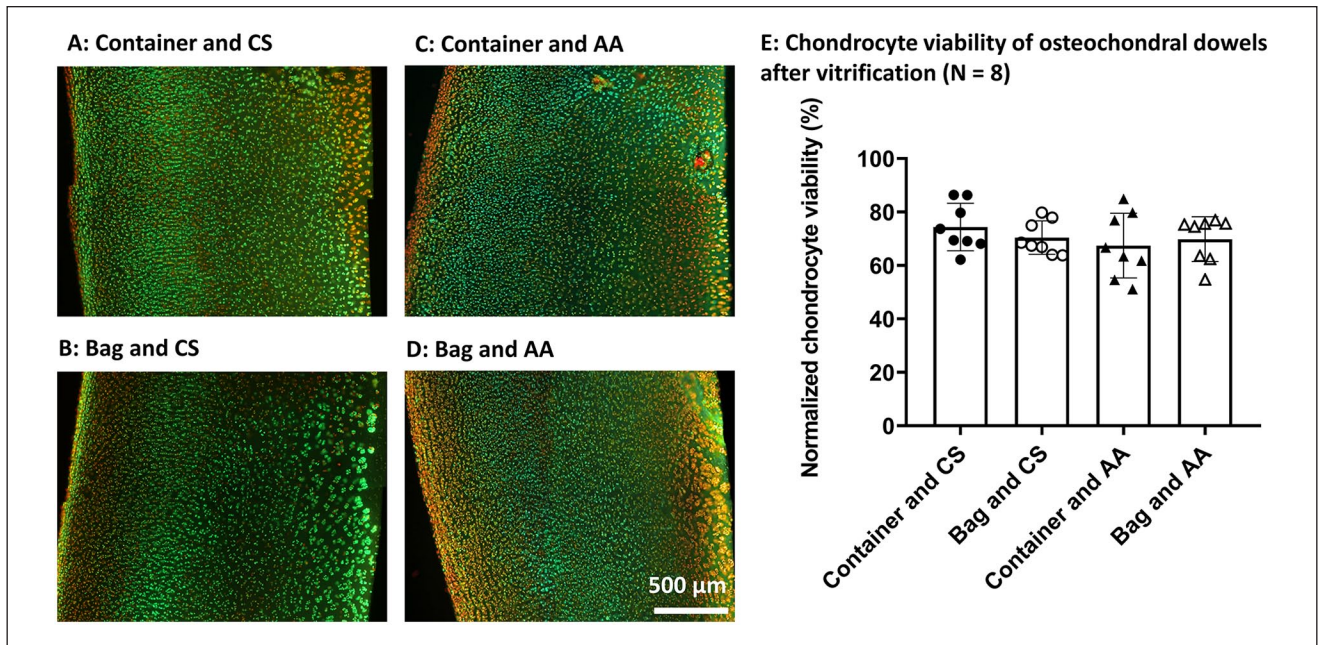
### Statistical Analysis

The numerical data are presented as mean  $\pm$  standard deviation (SD) of the results. The equality of variances for experimental variables was determined by Levene's test before multiple comparisons. Analysis of variance (ANOVA) with post hoc tests (Tukey test) or nonparametric tests (Mann-Whitney  $U$  test) was performed for multiple comparisons on sample cell viability or metabolic activity based on the sample configuration for different experimental conditions. The numerical data were analyzed using SPSS 20.0 software for statistical significance and figures were plotted with GraphPad prism 8.0 software. The  $P$  values are reported in the results or figure legends, statistical significance in the figures was indicated with asterisks respectively: \* indicates  $P < 0.05$ , \*\* indicates  $P < 0.01$ .

## Results

### Chondrocyte Viability of 10-mm Diameter Osteochondral Dowels Using 2 Packaging Methods

Representative fluorescent images of cartilage slices from osteochondral dowels among the 4 experimental groups after vitrification are shown in **Figure 5A to D**. As shown in **Figure 5E**, for the CS group, the normalized chondrocyte viability of osteochondral dowels stored with vitrification solution in a Falcon tube was  $74.4 \pm 8.9\%$  (mean  $\pm$  SD), while the normalized chondrocyte viability in the osteochondral dowels stored without vitrification solution in a bag was  $70.4 \pm 6.3\%$ . For the AA group, the normalized



**Figure 5.** Chondrocyte viability of 10-mm diameter osteochondral dowels after vitrification using 2 storage methods with (in a Falcon tube) or without (in a bag) a surrounding vitrification solution. **(A)** A representative image from an osteochondral dowel in the CS group after vitrification with surrounding cryoprotectants in a container (Note: Green stain indicates cell-membrane-intact and viable chondrocytes, red and yellow stains indicate cell-membrane-ruptured and dead chondrocytes). **(B)** A representative image from an osteochondral dowel in the CS group after vitrification without surrounding cryoprotectants in a bag. **(C)** A representative image from an osteochondral dowel in the AA group after vitrification with surrounding cryoprotectants in a container. **(D)** A representative image of an osteochondral dowel in the AA group after vitrification without surrounding cryoprotectants in a bag. **(E)** Quantification of chondrocyte viability of osteochondral dowels after vitrification between 2 storage methods in 2 additive groups.

chondrocyte viability of osteochondral dowels stored with vitrification solution in a tube was  $67.4 \pm 12.1\%$ , and the normalized chondrocyte viability in the osteochondral dowels stored without vitrification solution in a bag was  $69.8 \pm 8.4\%$ . There were no statistically significant differences between the storage with or without vitrification solution and the usage of CS or AA as additives during the vitrification procedure ( $P > 0.05$ ).

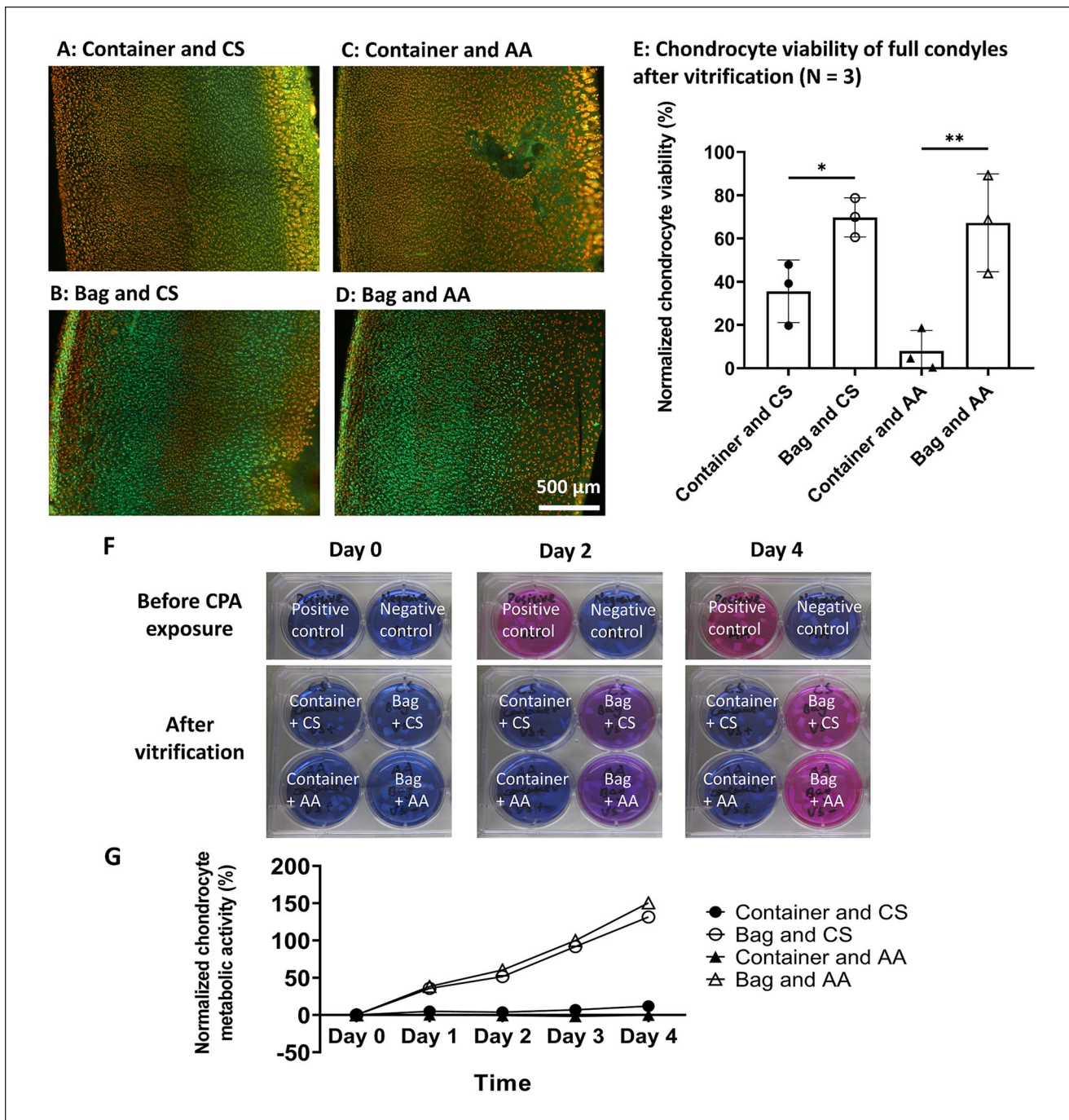
#### Chondrocyte Viability and Metabolic Activity of Full Femoral Condyles after Vitrification Using 2 Packaging Methods

Representative fluorescent images of cartilage slices from full femoral condyles among the 4 experimental groups after vitrification are shown in **Figure 6A to D**. As shown in **Figure 6E**, for the CS group, the normalized chondrocyte viability of full femoral condyles stored without vitrification solution in a bag ( $69.8 \pm 9.0\%$ ) was significantly higher than the normalized chondrocyte viability of the full femoral condyles stored with vitrification solution in a plastic container ( $35.6 \pm 14.4\%$ ;  $P = 0.03$ ). For the AA group, the normalized chondrocyte viability of full femoral condyles stored without vitrification solution in a bag

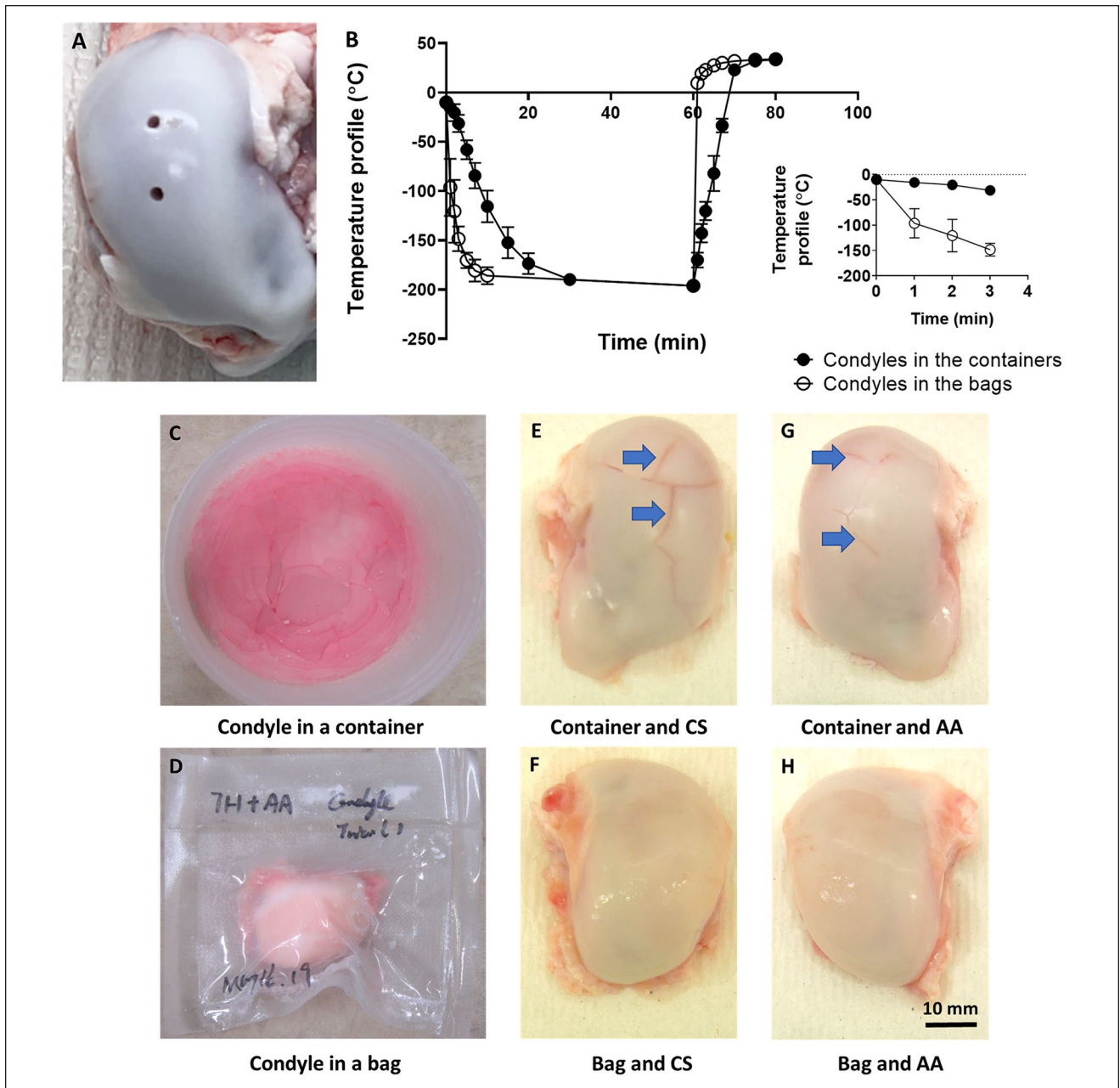
( $67.3 \pm 22.7\%$ ) was significantly higher than the normalized chondrocyte viability of the full femoral condyles stored with vitrification solution in a plastic container ( $7.9 \pm 9.6\%$ ;  $P = 0.01$ ). Representative images of chondrocyte metabolic activity of cartilage post vitrification of full femoral condyles among the 4 experimental groups from Day 0 to Day 4 are shown in **Figure 6F**. After cartilage treatment with CS or AA and vitrification in a bag without vitrification solution, the viable chondrocytes showed an active metabolic function at Day 4 similar to the positive control group. However, chondrocytes from full femoral condyles vitrified in a container showed no cellular activity in both groups treated with either CS or AA, similar to the negative control group. In addition, a similar normalized chondrocyte metabolic activity was seen in groups treated with CS or AA when using the same storage method (either stored in a container or stored in a bag) during the vitrification procedure (see **Fig. 6G**).

#### Temperature Profile of Full Femoral Condyles during the Vitrification and Warming Processes

A representative image of a full condyle with 2 positions on the cartilage surface for temperature measurement is



**Figure 6.** Chondrocyte viability and metabolic activity of full femoral condyles after vitrification using 2 packaging methods. **(A)** A representative image from a full condyle in the CS group after vitrification with surrounding cryoprotectants in a container (Note: Green stain indicates cell-membrane-intact and viable chondrocytes, red and yellow stains indicate cell-membrane-ruptured and dead chondrocytes). **(B)** A representative image from a full condyle in the CS group after vitrification without surrounding cryoprotectants in a bag. **(C)** A representative image from a full condyle in the AA group after vitrification with surrounding cryoprotectants in a container. **(D)** A representative image from a full condyle in the AA group after vitrification without surrounding cryoprotectants in a bag. **(E)** Quantification of chondrocyte viability of full femoral condyles after vitrification showing differences between 2 storage methods in the 2 additive groups. **(F)** Representative alamarBlue images of chondrocyte metabolic activity in full femoral condyles after vitrification with (in a container) or without (in a bag) a surrounding vitrification solution. **(G)** Normalized chondrocyte metabolic activity of the 4 experimental groups after vitrification followed by the 4-day alamarBlue assessment.



**Figure 7.** Temperature profile during cooling/warming and gross morphology of porcine femoral condyles after vitrification. **(A)** Two points on the porcine condyle cartilage surface for temperature measurement. **(B)** The temperatures of full femoral condyles ( $N = 3$ ) when vitrified with (in a container) or without (in a bag) a surrounding vitrification solution during plunge into liquid nitrogen followed by warming back to  $37^{\circ}\text{C}$  in water bath. Inset shows expanded view of temperature data within the first 3 minutes. **(C)** A condyle vitrified with surrounding cryoprotectants in a plastic container. **(D)** A condyle vitrified without surrounding cryoprotectants in a sealed vacuumed freezing bag. **(E)** Gross appearance of a post-vitrified condyle from the container and CS group (Blue arrows: cartilage fracture). **(F)** Gross appearance of a post-vitrified condyle from the vacuumed freezing bag and CS group. **(G)** Gross appearance of a post-vitrified condyle from the container and AA group (Blue arrows: cartilage fracture). **(H)** Gross appearance of a post-vitrified condyle from the vacuumed freezing bag and AA group.

shown in **Figure 7A**. Temperatures during cooling and warming of full femoral condyles vitrified with (in a container) or without (in a bag) a surrounding vitrification solution during plunge into liquid nitrogen ( $-196^{\circ}\text{C}$ )

followed by warming to  $37^{\circ}\text{C}$  in a water bath are shown in **Figure 7B**. The temperature of full femoral condyles stored in a bag reached below  $-150^{\circ}\text{C}$  between 2 and 3 minutes after plunge into liquid nitrogen, while full femoral



condyles stored in a container took more than 15 minutes to reach below  $-150^{\circ}\text{C}$ . During the warming process, the full femoral condyles stored in a bag reached above  $0^{\circ}\text{C}$  within 1 minute, compared to full femoral condyles stored in a container that took more than 7 minutes to reach  $0^{\circ}\text{C}$ .

### Gross Morphology of Full Femoral Condyles after Vitrification

Morphologies of full femoral condyles after vitrification and warming are shown in **Figure 7C to H**. The full femoral condyles were either stored with surrounding CPAs in a plastic container (**Fig. 7C**) or stored without surrounding CPAs in a vacuumed bag (**Fig. 7D**). After warming and CPA removal, condyles stored with vitrification solution in the containers (either treated with CS, **Fig. 7E**, or AA, **Fig. 7G**) demonstrated visible fractures in the cartilage surface (indicated by blue arrows) compared to those condyles stored without vitrification solution in vacuumed bags (either treated with CS, **Fig. 7F**, or AA, **Fig. 7H**).

### Discussion

Cryopreservation by vitrification is a promising technology to preserve cells and tissue for long term without ice crystal formation, and it can be achieved by 2 approaches<sup>22</sup>; the first approach is an equilibrium approach which involves permeating high concentrations of CPA into the cells and matrix, and the second approach is a nonequilibrium approach which involves cooling the sample fast enough to outrun ice formation and kinetically avoid ice crystal formation. Both approaches require permeating large amounts of CPAs into the tissue. CPA permeation is a main factor that determines whether the vitrification of articular cartilage tissue will be successful. Shardt *et al.*<sup>18</sup> proposed an optimized 7-hour CPA permeation protocol based on an engineering model incorporating predictions of CPA concentration, freezing point, and tissue vitrifiability. In the current study, we found that after applying the optimized 7-hour CPA permeation protocol, the chondrocyte viability after vitrification in small osteochondral dowels (10-mm diameter on a bone base) showed approximately 70% chondrocyte viability after vitrification for both storage in a Falcon plastic tube surrounded with CPAs and storage in a vacuumed bag without CPAs (**Fig. 5E**). These results regarding cell membrane integrity on 10-mm diameter porcine osteochondral dowels with 2-mm thick articular cartilage after vitrification are consistent with our previous findings (~75%) using the same osteochondral dowel size of human articular cartilage.<sup>14</sup> Our results are comparable to Brockbank *et al.*'s best findings in the 6-mm diameter porcine femoral cartilage plugs after vitrification using alamarBlue assay for chondrocyte assessment.<sup>12</sup> Next, we found a similar chondrocyte

viability (~65% to 70%) in the full femoral condyles for which the surrounding CPAs were removed from the containers before plunging into liquid nitrogen (**Fig. 6E**). These results indicate that the 7-hour CPA permeation strategy<sup>18</sup> is applicable to the vitrification of full condyle articular cartilage tissue.

Importantly, we found no statistically significant differences in the chondrocyte viability of 10-mm diameter osteochondral dowels between the storage in a Falcon plastic tube with surrounding CPAs and storage in a vacuumed bag without surrounding CPAs (**Fig. 5E**). This result indicated that even if the surrounding CPAs are removed from around the osteochondral tissue during cooling to  $-196^{\circ}\text{C}$ , the permeated CPA in the articular cartilage is sufficient to protect the chondrocytes from freezing injuries. Thus, we addressed the gaps in the literature to show that a surrounding CPA solution is not required for vitrification of articular cartilage. This is contrary to the current cryopreservation practices, in which articular cartilage is immersed in a certain amount of CPA solution for vitrification before cooling below  $-130^{\circ}\text{C}$ .<sup>11,12,14,23</sup> We propose that high CPA concentration within the matrix is sufficient to transform the tissue into a solid without ice formation when the articular cartilage is cooled sufficiently rapidly to cryogenic temperatures.

Following the CPA permeation, articular cartilage is usually kept in a high concentration CPA solution<sup>12-14</sup> and plunged into liquid nitrogen at  $-196^{\circ}\text{C}$  for vitrification. The requirement of storing articular cartilage tissue in extra CPA solution for vitrification has not been investigated. Scaling up the vitrification process for increased tissue size faces the challenge of decreased cooling and warming rates. The long time required to warm articular cartilage from a large vitrified glass (e.g., intact femoral condyle surrounded by a correspondingly large amount of vitrification solution) at  $-196^{\circ}\text{C}$  to  $37^{\circ}\text{C}$  may affect chondrocyte survival due to devitrification.<sup>24</sup> In addition, nonuniform temperatures may occur throughout the sample during cooling and warming,<sup>25,26</sup> which can cause tissue cracks or fractures.<sup>27</sup> Kroener and Luyet<sup>28</sup> reported observations of glycerol solution forming cracks during the vitrification process. Stolberg-Stolberg *et al.*<sup>29</sup> showed that fractures in cartilage allograft can increase the release of inflammatory markers that further impact the chondrocyte metabolic activity and viability via apoptosis. Cracking was not significant in the 10-mm diameter osteochondral dowels; this is probably due to the small volumes of osteochondral tissue. However, cracking became a challenge when scaling to full femoral condyles where cartilage fractures were noted on the surface after vitrification with a surrounding CPA solution (**Fig. 7E and G**). Temperature profiles within the articular cartilage during the different methods of vitrification and warming are shown in **Figure 7B**. The cooling and warming rates of full femoral condyles stored in vacuumed bags without surrounding CPAs were faster than those condyles stored in plastic containers with surrounding

CPAs. Having a solution surrounding the cartilage tissue is disadvantageous not only because it induced thermal cracking to the cartilage, but also because it slowed down the cooling and warming of the cartilage that may lead to tissue devitrification and chondrocyte death. From the above findings, we concluded that removing the surrounding CPAs before vitrification can (1) improve the cooling and warming of articular cartilage (**Fig. 7B**) and (2) reduce the thermal-mechanical stress in the large volume that results in cracks propagating through the articular cartilage (**Fig. 7E-H**), and this helps retain a high chondrocyte viability (**Fig. 6E**) and functionality (**Fig. 6G**).

There are other approaches to reduce thermal stress in the literature. Pegg *et al.*<sup>27</sup> proposed an approach to avoid thermal stress in arteries by controlling the cooling and warming rates. However, this approach required careful calculation of the thermal properties of the vitrification solution and the targeted sample. Rabin *et al.*<sup>26,30,31</sup> further developed a mathematical model to calculate the thermal-mechanical stress during the freezing of biological samples, for example, rabbit liver, kidney, and brain. More investigation is required to quantify and understand the thermal-mechanical effects in articular cartilage when it undergoes vitrification/warming processes. Our current approach is simple and successful in preventing cartilage fractures caused by the surrounding CPAs and is effective in improving the vitrification outcomes of articular cartilage.

Chondroitin sulfate was used as an additive for the vitrification of both human<sup>14</sup> and porcine<sup>13</sup> articular cartilage to protect chondrocytes from the toxic effects of high concentrations of CPA in our previous works. Inclusion of ascorbic acid was able to maintain a similar high chondrocyte survival compared to the inclusion of chondroitin sulfate during the CPA permeation and vitrification processes (see **Fig. 5** and **Fig. 6**). Ascorbic acid reduced reactive oxygen species during the CPA permeation process and protected chondrocytes from oxidative stress in recent research.<sup>32</sup> The use of ascorbic acid in the vitrification of articular cartilage is a promising alternative to chondroitin sulfate for future investigations.

Although the optimized approach has shown to be promising in vitrification of porcine femoral condyles, there are limitations in the current study. This study did not investigate the mechanical properties of post-vitrified cartilage, which is an important aspect of cartilage functionality, regardless that cell viability is the current primary method used for assessment of cartilage recovery after cryopreservation. Chondrocyte viability after vitrification was approximately 70%. That result can be improved upon to obtain an enhanced clinical outcome with further protocol modification. *In vitro* results obtained from this study require validation *in vivo* using an animal model as well as human tissue before protocol translation into clinical use.

In summary, this is the first study demonstrating the vitrification of porcine full femoral condyles, indicating the possibility to scale up the vitrification of articular cartilage from small osteochondral dowels to full-size femoral condyles. After tissue vitrification and subsequent warming, our results showed similar chondrocyte viability of post-vitrified 10-mm diameter osteochondral dowels when stored with or without a surrounding vitrification solution, which confirmed that articular cartilage can be vitrified with sufficient CPA permeation in the absence of a vitrification solution surrounding the tissue. In addition, higher chondrocyte viability and metabolic activity can be maintained in full femoral condyles when stored without a surrounding vitrification solution when compared to those with a surrounding vitrification solution. This difference is due to faster cooling/warming rates and less thermal-mechanical stress on the large volume of articular cartilage tissue. This study provides guidance for the development of articular cartilage packaging processes for vitrification and will benefit tissue banking of intact human articular cartilage.

#### Author Contributions

KW, LL, JAW, and NMJ were responsible for the conception and design of the experiments. KW and LL performed the experiments, data acquisition, data analysis, and quality assessment. KW, LL, JAW, and NMJ contributed to the interpretation of experimental results. KW wrote the first draft of the manuscript with guidance from JAW and NMJ. All authors reviewed and approved the final manuscript to be submitted.

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#### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: NMJ and JAW are co-inventors on US Patent 8,758,988 and Canadian Patent 2,788,202: Jomha NM, McGann LE, Elliott JAW, Law G, Forbes F, Torgabeh AA, Maghdoori B, Weiss A. "Cryopreservation of articular cartilage," University of Alberta, 2014. KW and LL have no conflicts of interest to disclose.


## Ethical Approval

The use of animal tissue for research was approved by the Research Ethics Office at the University of Alberta (RES0036331).

## Animal Welfare

Guidelines for humane animal treatment did not apply to the present study because porcine joints were collected from a slaughterhouse after slaughtering for commercial consumption and no animals were specially sacrificed for this research project.

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