CRYOPRESERVATION OF THIN OCA PROCHONDRIX® CR SUPPRESSES APOPTOSIS AND MAINTAINS VIABILITY COMPARED TO 14-DAY REFRIGERATED STORAGE

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PURPOSE

Viable chondrocytes in osteochondral allografts (OCAs) have been shown to be necessary for positive clinical outcomes in articular cartilage health. Traditionally, fresh OCAs aren't available until 14 days after procurement and have a limited shelf-life whereas cryopreserved grafts are preserved immediately. This study characterizes the viable cell population of cryopreserved and refrigerated samples by assessing Caspase-3 signaling and apoptosis to evaluate the impact of storage conditions on programmed cell death. A cryopreservation method that can both reduce the impact to the cellular viability and cellular pathway towards apoptosis indicates a successful way to preserve articular cartilage allografts, while maintaining their native functional properties.

METHODS

Cartilage samples were collected from 5 cadaveric human donors.

ProChondrix CR allografts were prepared and cryopreserved using a proprietary process with a commonly used cryoprotectant and stored at -80˚C for 14 days.

Refrigerated samples were prepared and aseptically packaged in media and stored for 14-days at 4˚C.

Fresh (T=0) samples were processed immediately.

All cartilage samples were enzymatically digested using collagenase and filtered (100µm strainer) and centrifuged (500g, 5 min.).

Cell pellets were resuspended in Chondrocyte Growth Medium (Cell Applications, San Diego, CA), diluted with trypan blue stain. Viable cells were counted using the countless automatic cell counter (Invitrogen, Carlsbad, CA).

For apoptotic cell characterization, samples were concentrated (1x10^6 cells/mL), washed with binding buffer (1mL), resuspended (100µL of binding buffer), stained with the apoptosis kit (Invitrogen, Carlsbad, CA), and incubated at 37˚C for 15 min.

Samples were resuspended in 400 µL of buffer, read, and analyzed with a NovoCyte Flow Cytometer (Agilent Technologies, Santa Clara, CA). The apoptotic, viable/living, and necrotic/dead populations, respectively, were recorded.

Tissue was imaged with immunofluorescent imaging to detect Caspase-3, a marker for apoptosis.

2022/ICRS
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RESULTS

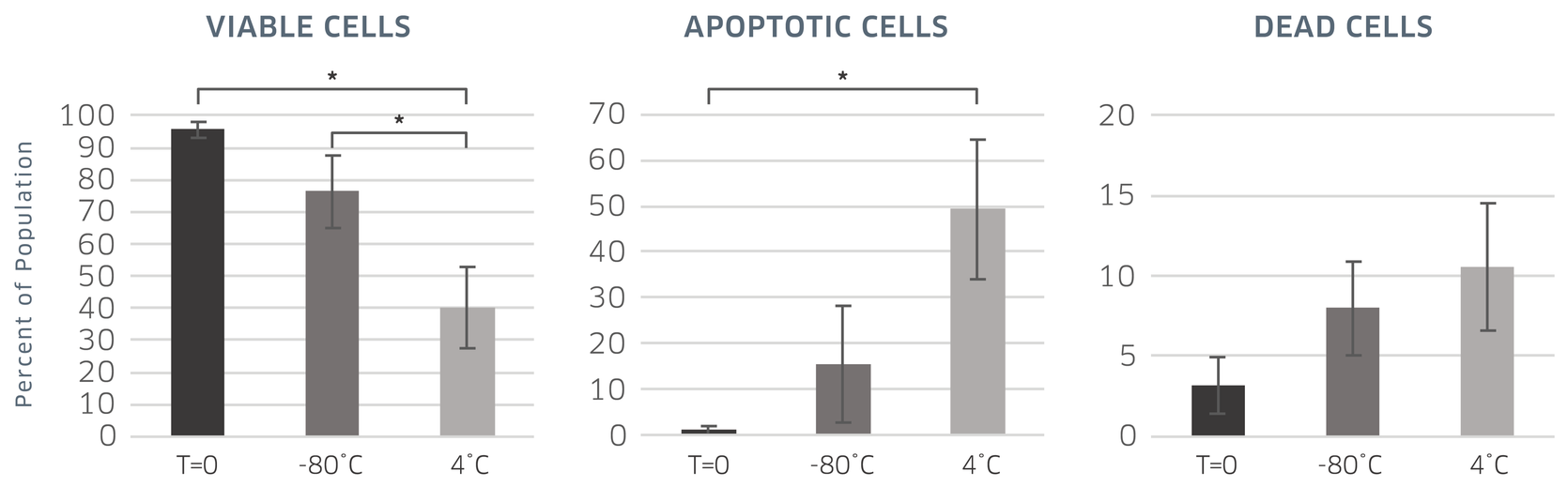
CELLULAR CHARACTERIZATION USING FLOW CYTOMETRY

The samples that were run on the flow cytometer were stained and analyzed to assess their apoptotic, necrotic and living populations. These three populations were seperated out below and compared to the apparent viability or the sum of the apoptotic and living populations. The apparent viability reflects a viability that would be generated using traditional trypan blue viablity methods. Characterization using flow cytometry shows that cryopreserved and fresh cartilage have a high cell viability and low percentage of apoptotic cells (**Figure 1**). When compared to refrigerated samples, cryopreservation matinains a higher population of viable cells, with fewer apoptotic cells. The percent of viable cells of refrigerated samples was significantly lower than both initial fresh and cryopreserved samples.

CASPASE-3 IMMUNOFLUORESCENT IMAGING OF PROCHONDRIX CR

Immunofluorescent imaging was employed to selectively stain for the Caspase-3 protein found in apoptotic cells. The samples from each group were stained for Caspase-3 protein and counterstained with a nuclear stain. The imaging showed Caspase-3 expression to be lowest in fresh cartilage and highest in refrigerated samples, shown in (**Figure 2**). The intensity of the Caspase-3 staining also corresponds with the apoptosis data collected using flow cytometry.

FIGURE 1



CONDITION	APPARENT VIABILITY†	PERCENT VIABLE	PERCENT APOPTOTIC	PERCENT DEAD
Fresh Control; T=0 (N=5)	96.82 (±1.74)	95.75 (±2.58)	1.07 (±0.89)	3.18 (±1.74)
Cryopreserved -80˚C; T=14 (N=5)	92.02 (±2.92)	76.52 (±11.30)	15.50 (±12.67)	7.98 (±2.92)
Refrigerated 4˚C; T=14 (N=5)	89.42 (±3.96)	40.13 (±12.82)	49.30 (±15.30)	10.58 (±3.96)

FIGURE 2

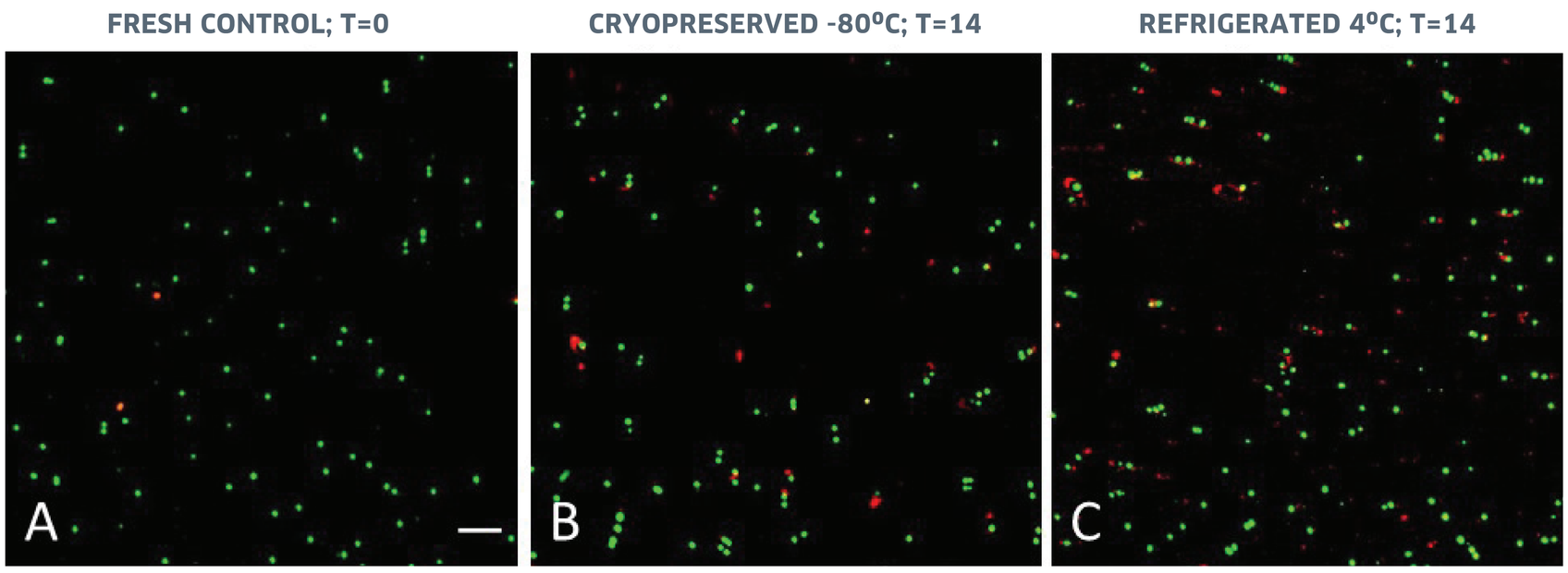


Figure 1: The mean values of each storage condition for apoptotic, living and dead/necrotic cells; error bars represent the standard error of the mean (SEM) and are shown below in parentheses. *Indicated p value <0.05 using a paired t-test between groups. †Apparent viability calculated by combining viable and apoptotic cell populations, representing a viability measurement based on membrane integrity. **Figure 2:** Immunofluorescent Caspase-3 expression in fresh control (A), cryopreserved (B), and refrigerated storage (C); Caspase-3 expression shown in red with cell nuclei in green, 10X magnification with 10-micron scale bar.

CONCLUSION

Cryopreserved ProChondrix CR overall shows a higher cell viability and fewer apoptotic cells compared to refrigerated storage. After 14 days of refrigeration, more than half of the viable cells were apoptotic. This suggests that cryopreservation of thin OCAs may provide an alternative solution to refrigerated storage, allowing more patients to receive allografts due to the increased shelf life (2 years) that cryopreservation allows. This would help alleviate the common issue of tissue availability as it limits the constraints that come with fresh tissue allografts, such as a limited shelf-life.

References available online in individual abstracts – https://cartilage.org/icrs2021-world-congress-berlin/scientific-programme/