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# LASER-ETCHED CRYOPRESERVED OSTEOCHONDRAL ALLOGRAFT, PROCHONDRIX® CR MAINTAINS METABOLICALLY ACTIVE AND VIABLE CELLS AFTER TWO YEAR STORAGE

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# Laser-Etched Cryopreserved Osteochondral Allograft, ProChondrix® CR Maintains Metabolically Active and Viable Cells After Two Year Storage

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## INTRODUCTION

Fresh osteochondral allografts (OCA) have been used for decades to repair articular cartilage defects. There is a limitation to the use of fresh OCA due to short shelf life (28-35 days) and size matched donor requirements.[1,2] AlloSource's thin OCA, ProChondrix, is a living-cell, intact, fresh cartilage matrix processed from adult donors (16-39 years old) which utilizes laser etching located on the deep side to enhance cell signaling and chondrocyte migration.[3] ProChondrix can be used without specific site donor size matching but still has the same shelf life constraints. There was a need to develop alternative storage procedures, including cryopreservation of OCA, to overcome these limitations. Conventional cryopreservation methods utilize a cryoprotectant and a controlled rate freezer to slow the cooling process to prevent ice crystal formation and subsequent cell damage. However, an effective cryopreservation method utilizing conventional techniques remained limited for OCA because the cryoprotective agents could not successfully penetrate through the graft.[4] AlloSource has developed a proprietary method incorporating conventional techniques along with a method which sufficiently loads the cryoprotectant within the cartilage matrix to successfully cryopreserve the chondrocytes in the OCA, ProChondrix® CR, without compromising cell viability. It is therefore necessary to characterize the effect of cryopreservation on chondrocyte viability and functionality of ProChondrix CR allografts, in comparison to currently available fresh grafts. Graft functionality was determined through an explant study testing metabolic activity which was performed by affixing the allograft (using fibrin glue) to the bottom of culture plate wells, further mimicking the intended clinical application. In our earlier studies, we showed that fresh ProChondrix maintains high cell viability and various growth factors throughout processing and the entire shelf-life of 35 days.[3,5] The purpose of this study was to evaluate cell viability and metabolic activity of ProChondrix CR after 2 years of frozen storage following cryopreservation. Furthermore, ProChondrix CR was compared to two controls; 1. Fresh (non-cryopreserved) ProChondrix at its 35-day expiration date, and 2. Fresh ProChondrix after 14 days of refrigerated storage. The two controls were chosen to encompass the earliest date fresh cartilage grafts can be released for implantation due to the necessary microbial testing that is required for product release and its expiration date.

## MATERIALS AND METHODS

### PREPARATION OF PROCHONDRIX CR

All ProChondrix CR (AlloSource, Centennial, CO) grafts were recovered from research consented cadaveric human donors, between 16 and 39 years of age, and prepared at a diameter of 11 mm, 1 mm thickness and laser-etched with a 1.5 mm square pattern. Samples were prepared using AlloSource's proprietary cryopreservation process. Samples were stored in a -80˚C freezer for a minimum of 2 years. All samples were thawed in a 37˚C water bath and subsequently extracted from the storage vial. Any residual cryopreservation medium was removed by a simple rinse with saline.

### VIABILITY AND METABOLIC ACTIVITY STUDIES OF PROCHONDRIX CR

The cell viability of cryopreserved grafts was performed following collagenase digestion (50 mg Collagenase Type I (MediaTech, Manassas, VA), 100 mg Collagenase Type II (Life Technologies, Waltham, MA) and 50 mL of Chondrocyte Growth Media (CGM)) and incubated at 37˚C for 16-24 hours. Following the digestion, samples were filtered through a 100µm strainer, then spun at 500G for 5 minutes. Cell pellets were then resuspended in 2 mL CGM. This cell solution was then utilized in the viability studies via trypan blue. For trypan blue, an aliquot of the cell solution obtained in the digestion protocol was diluted with trypan blue stain (Invitrogen, Carlsbad, CA). This solution was then read using Countess automatic cell counter (Invitrogen, Carlsbad, CA) using the Countess disposable hemocytometers.

For metabolic activity studies, TISSEEL (Baxter, Deerfield, IL) was utilized to secure the cryopreserved grafts (stored for 2 years), applied to the bottom of a 12 well plate with grafts to then be inserted on top. Explanted grafts were cultured under standard conditions for 12 weeks. To observe the metabolic activity of the explanted grafts, a 10% Presto Blue viability stain (Life Technologies, Waltham, MA) in CGM was added to each sample and incubated for 3 hours at 37˚C. A 100µL aliquot of each sample was then read on a plate reader against a standard curve consisting of cultured chondrocytes at a wavelength of 535, 615 nm.

# RESULTS AND DISCUSSION

## EXAMINATION OF VIABILITY OF PROCHONDRIX CR BY TRYPAN BLUE

Viability is the number of live cells as compared to the total number of cells in a graft. Viability of ProChondrix CR was determined by Trypan Blue stain, a frequently used assay to determine cell viability. Trypan Blue is an exclusion method as the live cells are left unstained, yet the dead cells are stained with a blue dye. The unstained and stained cells can then be counted under a microscope or an automated cell counter.

A 70% or higher viability of chondrocytes in OCA has been shown to increase positive clinical outcomes.[7] After two years of cryopreserved storage, ProChondrix CR maintained a viability of 94.97% compared to a viability of 87.50% for ProChondrix at its expiration of 35 days in refrigerated storage and a viability of 70.5% for other cryopreserved cartilage grafts currently on the market after 2 years of storage at -80˚C (Figure 1).[8]

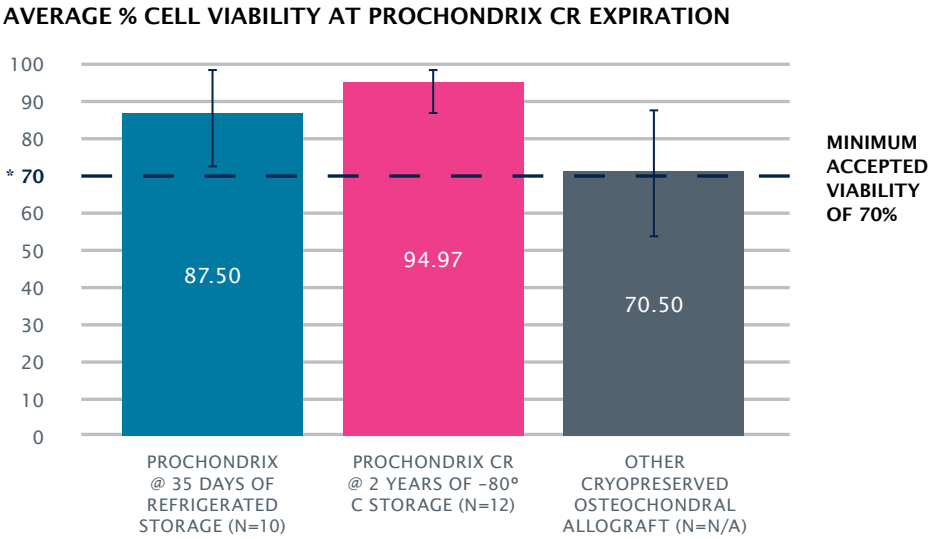


**Figure 1.** A comparison of viability using trypan blue of ProChondrix tested at 35 days of refrigerated storage, ProChondrix CR tested after 2 years of storage at -80˚C, and other cryopreserved grafts currently on the market.

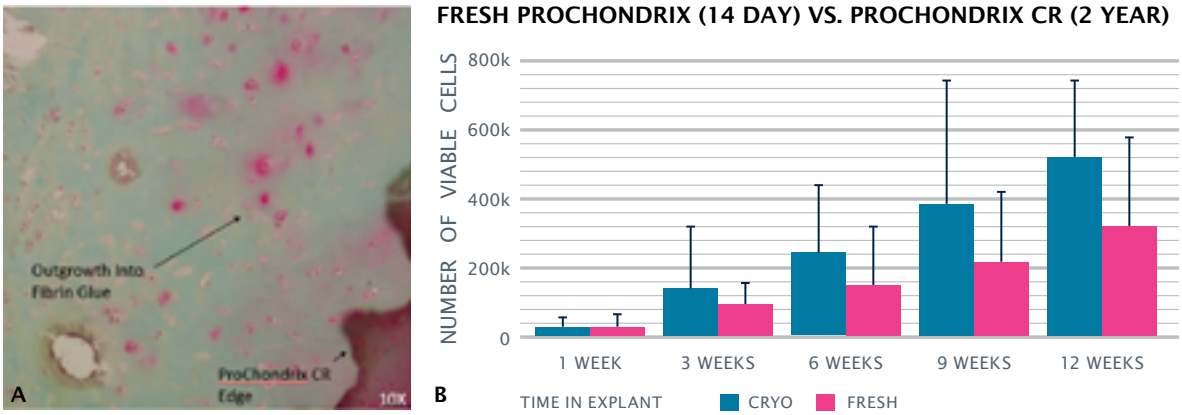


**Figure 2.** (A) Safranin-O stained outgrowth of cells from 12 weeks explanted ProChondrix CR, post-2 yrs. cryopreservation. (B) Presto Blue metabolic assay showed metabolically active cells with a steady growth over time for both fresh (stored for 14 days in media at 4˚C) and cryopreserved ProChondrix CR stored for 2 years at -80˚C.

Safranin-O staining was done on samples, after the 12-week explantation study period was finished, to evaluate the ability to proliferate and produce new Collagen II. Figure 2A displays cell outgrowth into the fibrin glue where new collagen II is present.

The metabolic activity of each explanted graft was tested using Presto Blue. Presto Blue uses the cell's reducing environment to produce a fluorescent dye and thus quantitatively measure viability.[9] The explantation of two-year cryopreserved ProChondrix CR compared to ProChondrix stored for 14 days in refrigerated storage resulted in the outgrowth of chondrocytes for both groups. While both groups were equivalent at the initial time point, the cryopreserved group maintained a higher metabolic activity between weeks 3 and 12, as seen in Figure 2B. A control of ProChondrix stored for 14 days in refrigerated storage was used for comparison because current OCA require a 14 day microbial screening panel to be released for transplant. This tissue will also represent the most metabolically active tissue available due to the short storage time.

## CONCLUSION

The data presented in these studies show that the long-term cryopreservation (2 years shelf-life) of ProChondrix® CR maintains viable, metabolically active cells. As shown in Figure 1, the total averaged viability based on trypan blue dye exclusion test, was found to be 94.97%. Graft functionality was determined by explanting each of the grafts using fibrin glue, and observing growth over a period of 12 weeks. It was determined that metabolically active cells, which displayed capabilities for cellular outgrowth, were found within all samples for ProChondrix CR (Figure 2B). Figure 2A displays Collagen II within the outgrowth of cells following 12 weeks of explantation, indicating the living chondrocytes' ability to grow out of the graft, proliferate and produce new collagen II.

Cryopreservation of ProChondrix mitigates the limited shelf life which consistently limits the availability of fresh osteochondral allografts to clinicians. AlloSource's proprietary cryopreservation technique utilizes a common cryoprotectant in a method which allows for the cryo-media to penetrate the depth of the cartilage matrix using a rapid mass transfer technique which has displayed no adverse effect on cell viability or metabolic activity. In addition, the data also suggests that the original composition and the graft integrity have been maintained throughout this novel cryopreservation process. ProChondrix CR maintains a high cellular viability up to 2 years, providing a readily available allograft solution that also includes metabolically active cells to aid in the regeneration and repair of articular cartilage.

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