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CRYOPRESERVED OSTEOCHONDRAL ALLOGRAFT, PROCHONDRIX® CR MAINTAINS METABOLICALLY ACTIVE, VIABLE CELLS

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AlloSource®, Centennial, CO

Cryopreserved Osteochondral Allograft, ProChondrix® CR Maintains Metabolically Active, Viable Cells

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Introduction

Fresh osteochondral allografts (OCA) have been used for decades to repair articular cartilage defects. There is a limitation in the use of fresh OCA due to short shelf life (35 days) and size matched donor requirements.[1,2] AlloSource®'s OCA, ProChondrix®, is a living-cell, intact, fresh cartilage matrix processed from adult donors which utilizes laser perforations 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 has been a need to develop alternative storage procedures, including cryopreservation of OCA, to overcome the aforementioned limitations. Conventional cryopreservation methods utilize a cryoprotectant and a controlled rate freezer to slow the cooling process in order to prevent ice crystal formation and subsequent cell damage. However, an effective cryopreservation method utilizing conventional techniques remains limited for OCA due to the fact that the cryoprotective agents cannot 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 in order to successfully cryopreserve an OCA, ProChondrix® CR, without compromising cell viability. It is therefore necessary to characterize the effect of cryopreservation on ProChondrix CR allografts, by analyzing the chondrocyte viability and functionality in comparison to fresh grafts. Graft functionality was determined through fixing the allograft using fibrin glue to the bottom of a well plate, further mimicking the intended clinical application. In our earlier studies, we have shown that fresh ProChondrix maintains high cell viability and various growth factor presence throughout the processing procedures and shelf-life.[3,5] The purpose of this study was to evaluate cell viability and metabolic activity of ProChondrix® CR after 6 months of cryopreservation, and to compare to historical controls of fresh ProChondrix.

Methods

PREPARATION OF PROCHONDRIX® CR

All ProChondrix CR (AlloSource®, Centennial, CO) grafts were recovered from cadaveric human donors, between 16 and 35 years of age, consented for research and prepared at a diameter of 13 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 180 days, the current maximum shelf life. Samples were then thawed in a 37˚C water bath, removed from the cryopreservation medium and placed in a 12 well plate with Chondrocyte Growth Medium (CGM) and placed into a 37˚C incubator before further testing.

VIABILITY AND METABOLIC ACTIVITY STUDIES OF PROCHONDRIX® CR

Digestion of the cryopreserved grafts was performed by incubating samples at 37˚C in a collagenase solution (50 mg Collagenase Type I (MediaTech, Manassas, VA), 100 mg Collagenase Type II (Life Technologies, Waltham, MA) and 50 mL of CGM) overnight. 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 FACS (fluorescently activated cell sorting) buffer. This cell solution was then utilized for viability studies via flow cytometry and trypan blue. For trypan blue, an aliquot of the cell solution obtained in the digestion protocol was diluted 1:1 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.

Fibrin glue (Baxter, Deerfield, IL) was utilized to explant the cryopreserved grafts, applied to the bottom of a 6 well plate with grafts to be then inserted on top. Explanted grafts were cultured under standard conditions for 9 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.

Separate 2 mm x 1 mm ProChondrix CR samples were explanted for microscopy. Samples were washed 1X with Phosphate Buffered Saline (PBS). A solution of 50% acetone/50% methanol was prepared and 3 mL were added to each well. Samples incubated at 4˚C for 15 minutes and then washed in PBS 3X. Nonspecific binding sites were blocked by adding 3 mL of 10% Fetal Bovine Serum (FBS)/PBS to each well and let incubate for 1 hour. The primary antibodies, Collagen II (Proteintech, Chicago, IL) and Connexin-43 (Abcam, Cambridge, UK), at a concentration of 1:200 in 1.5% FBS/PBS solution were used. Ki-67 Alexa Fluor 647 (BioLegend, San Diego, CA) at a concentration of 1:100 was added in the same solution, incubated overnight at 4˚C, in the dark and then washed 3X with PBS. A solution was made of each of the secondary antibodies, fluorescein isothiocyanate (FITC, Invitrogen, Carlsbad, CA) and tetramethylrhodamine isothiocyanate (TRITC, Abcam, Cambridge, UK), at a concentration of 1:100 in 1.5% FBS/PBS solution, and was then added to each of the sections and incubated for 2 hours at room temperature in the dark, and washed with PBS 3X. The stain 4',6-diamidino-2-phenylindole (DAPI) was added for 5 minutes at 4˚C, in the dark, washed with PBS 3X and imaged using confocal microscopy.

Results

EXAMINATION OF VIABILITY OF PROCHONDRIX® CR BY TRYPAN BLUE

Viability is the amount of live cells as compared to the total number of cells. Viability of ProChondrix CR was determined by Trypan Blue stain, a frequently used assay to determine cell viability and is considered to be 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. An automated cell counter outputs a viability percentage for each sample.[6,7]

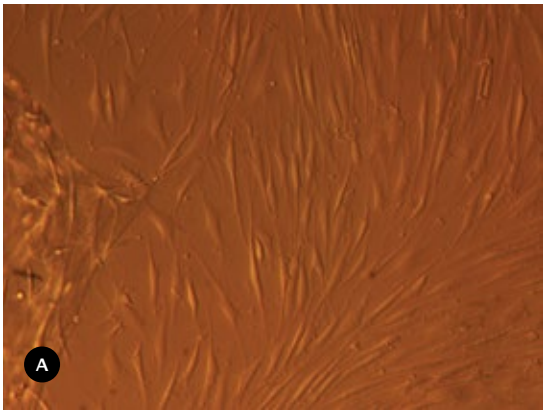
This output viability recording was read twice for each of the six samples, and the total average was concluded to be 88.83% ± 6.42%, as seen below in **Table 1**.

ProChondrix CR at 6 Month Expiration	n=6
% Viability Range	77.5-95%
% Viability Average	88.83%
Standard Deviation	6.42%

Table 1. Comparison of the viability of samples tested via trypan blue.

ANALYSIS OF METABOLIC ACTIVITY FOLLOWING EXPLANTATION OF PROCHONDRIX® CR

By utilizing fibrin glue to fix the graft to the bottom of a well plate, the intended clinical application would then be able to be assessed for functionality. Each sample was then subjected to standard culture conditions and an analysis of cellular metabolic activity over time. The outgrowth of cells was evident surrounding each of the explanted ProChondrix CR grafts post-cryopreservation and could be easily seen through light microscopy (**Figure 1A**). The image depicted was taken after 3 weeks of recovery and 3 weeks of explantation. The explantation of 6 months cryopreservation resulted in all donors displaying cell outgrowth resembling approximately 70-80% confluency in a six well dish, as seen in **Figure 1A**. This was also displayed while quantifying the metabolic activity of each explanted graft using Presto Blue. Presto Blue uses the cell's reducing environment to produce a fluorescent dye and thus quantitatively measures viability.[8] **Figure 1B** displays the metabolic activity of these explanted grafts following a course of 9 weeks. For this particular study, we compared fresh and cryopreserved ProChondrix utilizing different donors to compare the disparate processes. An upward growth was seen in both fresh ProChondrix samples and cryopreserved ProChondrix samples over the course of 9 weeks. Statistical significance was found at the 3 week time point between fresh and cryopreserved ProChondrix, but no statistical significance was found at 6 or 9 weeks between groups.



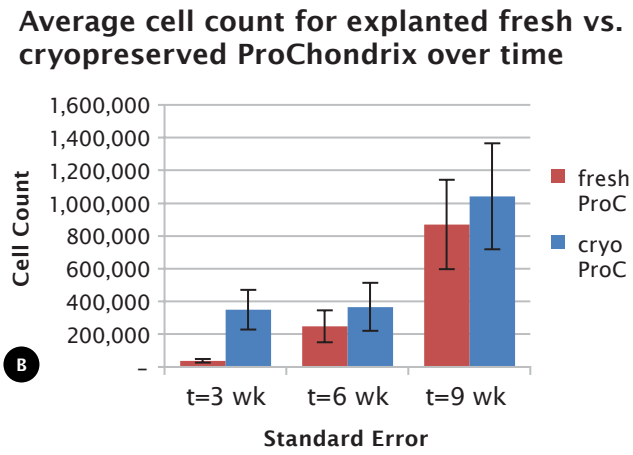


Figure 1. All ProChondrix grafts were explanted in six well plates using fibrin glue to secure graft to the bottom. **A:** Light microscopy image taken of outgrowth from a ProChondrix CR graft stored for 6 months. **B:** Tested for metabolic activity at various time points comparing fresh ProChondrix and ProChondrix CR grafts following a cryopreservation storage time of 6 months.

IMMUNOFLUORESCENCE STUIDES OF PROCHONDRIX® CR FOR CELLULAR OUTGROWTH AND MOBILITY, 12-WEEK POST-THAW

Cell outgrowth and mobility were displayed following the explantation of the ProChondrix grafts in fibrin glue. Cell outgrowth displays a vast amount of cell-cell interaction and communication in order to display directionality and mobility.[9] Gap junctions are channels which connect cells, allowing for them to communicate amongst each other and are comprised of specific proteins called Connexins.[4] This intercellular communication is immensely important in regulating normal cell function, tissue development and cellular motility.[10,11] It has been found that chondrocytes are mediated via Connexin-43 gap junctions, which have been shown to be correlated with cell motility.[11,12]

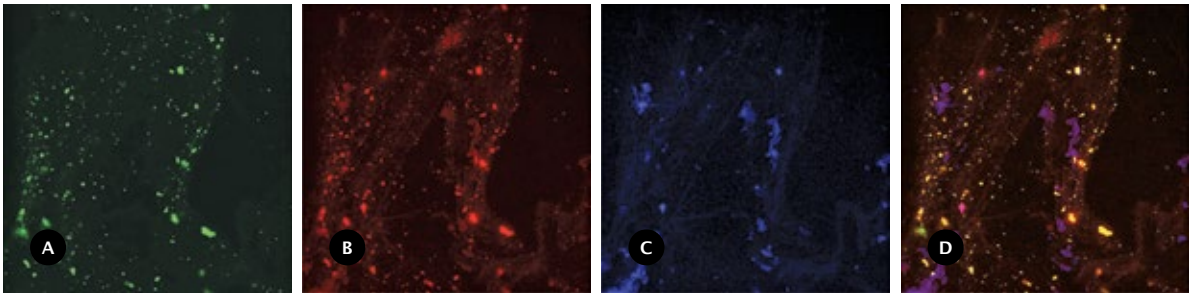


Figure 2. Confocal microscopy images taken at 10X of ProChondrix post-cryopreservation at 12 weeks post-explantation. **Image A)** displays Connexin-43 expression, **B)** Collagen II, **C)** DAPI (nucleus), and **D)** composite of the three channels.

Immunofluorescent staining qualitatively shows the various expression levels of Connexin-43 and Collagen II. It was found that the outgrowth of cells were shown to express Connexin-43 gap junctions, as seen in **Figure 2A**, as well as Collagen II expression, **Figure 2B**. This substantiates prior literature that cartilage is mediated through Connexin-43 gap junctions. It also displays the vast amount of cell-cell interaction which could be involved with cell motility. Collagen II deposits were also displayed within the outgrowth, **Figure 2B**, which may suggest chondrogenesis. **Figure 2C** shows the presence of nuclei, while **Figure 2D** represents a composite of **Figures A–C**.

Discussion

The data presented in these studies show that ProChondrix® CR maintains viable, metabolically active cells. The total averaged viability count from trypan blue was found to be 88.83%, as seen in **Table 1**. Graft functionality was determined by explanting each of the grafts using fibrin glue, further mimicking the intended clinical application, 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 cryopreserved ProChondrix **(Figure 1)**. **Figure 2** displays expression of gap junctions and Collagen II within the outgrowth of cells following 12 weeks of explantation, which can describe the amount of directionality and motility seen in each of the explanted ProChondrix CR grafts.

Cryopreservation of ProChondrix mitigates the limited shelf life which consistently limits the availability of fresh grafts. AlloSource's proprietary cryopreservation technique utilizes a common cryoprotectant in a method which allows for the cryo-media to breach the depth of the cartilage matrix, displaying no adverse effect toward cell viability. This data displays viable, metabolically active cells within each ProChondrix CR sample tested, further suggesting that the original composition of fresh ProChondrix has been maintained throughout cryopreservation process. This suggests that ProChondrix CR could be used as an effective option for cartilage defect repair.

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