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HIGH CHONDROCYTE VIABILITY OF PROCHONDRIX® AT THE MAXIMUM SHELF LIFE OF 35 DAYS: POTENTIAL FOR GOOD CLINICAL OUTCOME AND CONSISTENCY OF CARTILAGE ALLOGRAFT

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AlloSource®, Centennial, CO

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Introduction

The regeneration and restoration of hyaline cartilage through various surgical procedures has been presented with a variety of complications. Recent developments in the use of human allograft cartilage transplants have emerged as an alternative to older methods of joint damage repair. Such methods provide the benefit of transplanting an intact hyaline matrix to fill the gap produced by the surgical removal of damaged cartilage tissue. The presence of viable cells (chondrocytes) in allograft is important for successful engraftment, incorporation, recruitment, autocrine and paracrine effects. Upon transplantation, these cells continue to maintain a healthy graft matrix and provide the necessary signaling to recipient bone marrow cells to coordinate their response, potentially speeding up the time of recovery.[1] Based upon this assertion, a high and consistent viability of chondrocytes in allografts is needed to ensure a good clinical outcome is reliably achieved.

In recent years, several living cell cartilage grafts have been introduced into the market. Among the available grafts, ProChondrix® (AlloSource, Centennial, CO) and Cartiform® (Arthrex, Naples, FL) may be the most prominent options being adopted by surgeons. ProChondrix is a fresh chondral allograft with minimal manipulation of the cartilage matrix. Cartiform is a manipulated and enzyme treated chondral allograft, which is cryopreserved until the time of transplantation. The current available shelf life for fresh chondral allografts to maintain viable chondrocytes is 28 days postmortem when stored at 4˚C.[2] In order to ensure the maximum use of donor tissue, we examined the chondrocyte viability and shelf life of ProChondrix Cartilage Restoration Matrix allograft tissues. A series of well accepted experimental protocols were used in the study and the data indicates ProChondrix allograft has an average viability of 87.5 percent at the expiration of its 35-day shelf life.

Methods

The use of fluorescent staining has long been a standard method for assessing the viability of cells in allografts.[3] New research however, has indicated that this method highly overestimates the viability of chondrocytes in cartilage allografts.[4] Lightfoot et al have indicated that while approximately 75 percent of chondrocytes were observed to be viable with fluorescent staining, fewer than 30 percent of these cells actually contained nuclei, a direct indication they are not living cells. For this study we assessed the viability of chondrocytes in ProChondrix and its ability to outgrow and proliferate in culture, instead of fluorescent staining, at the maximum shelf life of 35 days.

1. Trypan blue, a viability exclusion dye test: ProChondrix grafts were digested with collagenase I and II for four hours at 37˚C on spinner flasks. The cell concentrate produced was then stained with trypan blue, a viability exclusion dye, and counted in a chamber slide with an automated cell counter.

2. Explant culture of ProChondrix allograft with fibrin glue: ProChondrix grafts were explanted into fibrin glue and the metabolic activity and ability of the chondrocytes in the graft to grow out over time was assessed through the use of a Presto Blue metabolic assay. The explants were tested at the time of explant start, then at three, six and nine weeks thereafter. Cultured chondrocytes were used to produce the standard curve for the test.

3. Immunofluorescent analysis for the presence of proliferating chondrogenic cells: After the final time point of the explant culture, grafts were collected and assessed with immunofluorescent staining for the presence of proliferating (Ki67) chondrogenic cells (collagen II). Cells expressing collagen II have been established to be chondrogenic. Ki67 is a well-known marker for proliferating cells and is completely absent in quiescent cells. The grafts and associated fibrin glue were fixed in formaldehyde for 48 hours and embedded in paraffin with a Thermo Scientific Shandon Citadel 2000. Serial sections were deparaffinized in Xylene and Antigen Unmasking was performed with 10 mm sodium citrate. The samples were then stained with Invitrogen Mouse Anti Ki67 FITC and ABCAM Rabbit Anti Collagen II. This primary incubation was performed overnight at 4˚C and a secondary staining was performed with ABCAM Goat Anti Rabbit Alexa Fluor 594. Slides were then stained with BioLegend DAPI. The final slides were imaged with a Nikon D-Eclipse C1 microscope.

Results

The cellular viability testing of ProChondrix after explant culture by fluorescence microscopy and Trypan blue staining for percent cellular viability showed an average 87.5 percent (range 73.5 to 99). Published data on Cartiform using fluorescent staining reported a 70.5 percent viability (range 54.5 to 88.5).[3] Results are summarized in **Table 1**.

Table 1: Percent viability of cells for ProChondrix and Cartiform.

	ProChondrix Trypan Blue	**Cartiform Fluorescent Cell Counting**[3]
% Viability Average	87.5	70.5
% Range of Viability	73.5 - 99	54.5 - 88.5

When expired ProChondrix grafts were explanted into fibrin glue they demonstrated the ability to increase their metabolic activity over time and thus, increase their active cell count. Initially, the graft explants were relatively metabolically inactive after being removed from storage, but by six weeks after explanation the amount of active cells per graft had increased to 45,000 and then 65,000 at nine weeks. This demonstrates that after 35 days of storage, the cells in ProChondrix are still healthy and active (**Fig. 1**).

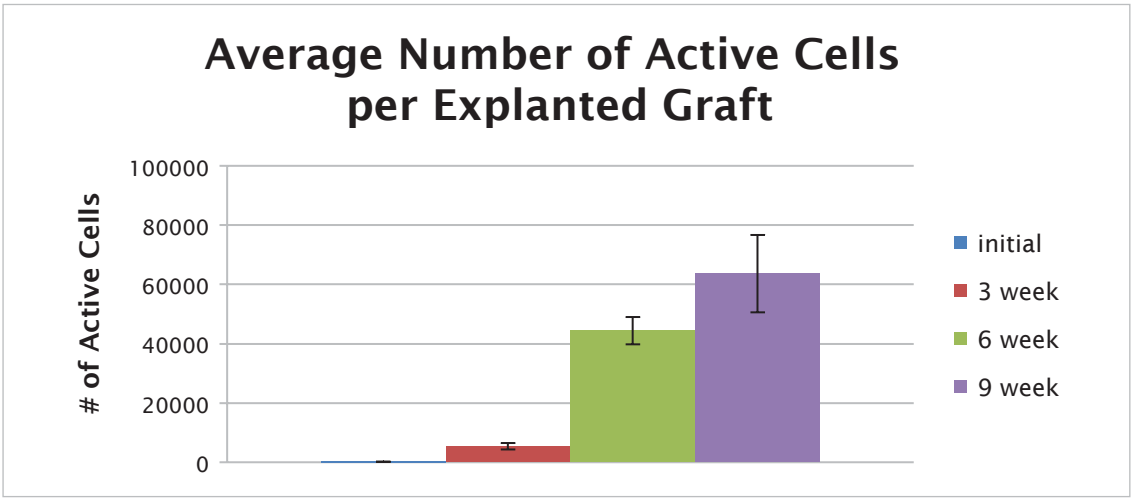

Figure 1. Average number of metabolically active cells in explanted ProChondrix from Presto Blue testing.

Fluorescent staining revealed chondrocyte proliferation in the explanted cultures (**Figure. 2**).

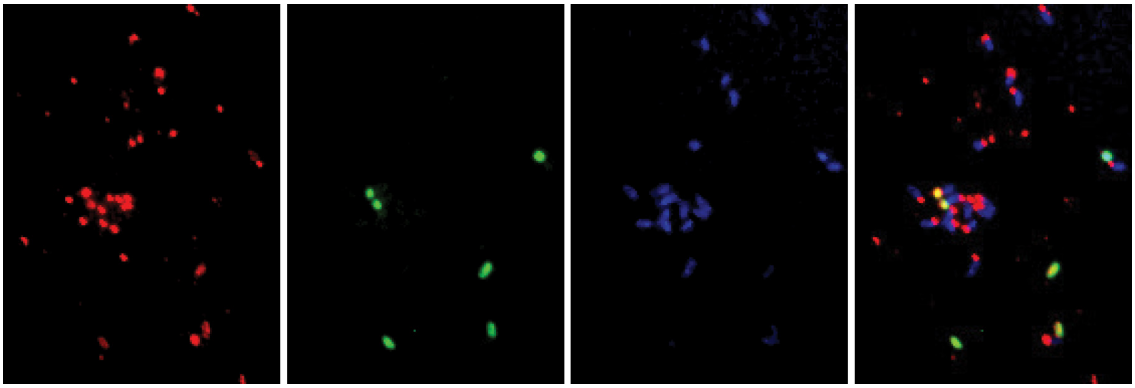

Figure 2. Immunofluorescent imaging of explanted ProChondrix. A: Collagen II (chondrocytes), B: Ki67 (proliferative cells), C: DAPI (nuclei), D: Combined.

The co-expression of collagen II and Ki67 proteins indicates the explanted cells are indeed proliferating chondrocytes. A broader image of the ProChondrix graft and the surrounding fibrin glue is shown in **Figure 3**.

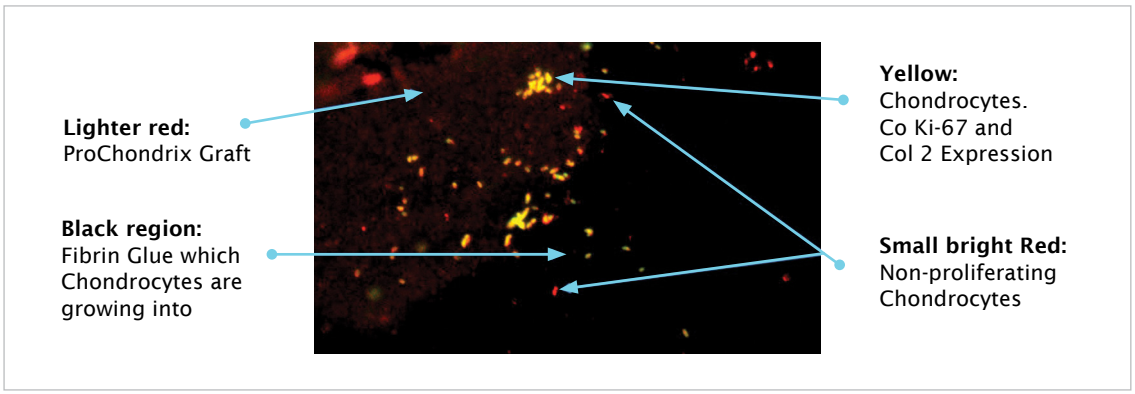

Figure 3. Immunofluorescence staining explanted ProChondrix. Red staining for collagen II. Green staining for Ki67.

This image demonstrates there are proliferating chondrocytes in the graft, as well as many that have migrated out of the graft into the surrounding fibrin glue. Not only did these cells maintain their proliferative capacity after 35 days of storage, but they retained their chondrocyte genotype. The lack of Ki67 expression in some chondrocytes also indicates this proliferation is proceeding in an orderly fashion, which is important to prevent excessive hyperplasia and outgrowth in a clinical setting.

Discussion

Currently, chondral defects are managed by microfracture or through the use of osteochondral allografts, the latter of which provide the possibility of transplanting large zones of cartilage for the replacement of defects.[5] However, the need for size matching and maintenance of fresh tissue viability has created a sizable shortage of these grafts for routine use. ProChondrix (fresh) and Cartiform (cryopreserved) have emerged onto the market as a viable alternative for chondral defects with no concomitant subchondral bone damage. To our knowledge, this is the first definitive study to compare the cell viability of these cartilage allografts. Prior studies using fluorescent staining to assess the viability of chondrocytes in cartilage tissue have displayed the potential for high false-positives. As such, more reliable explant cultures and immunophenotyping of proliferating chondrogenic cells in the allograft are critical in order to assess the true quality of the grafts.

The importance of the viability of cells has been established by Cook et al[1] in chondral allografts with a ≥70 percent value being absolutely required for any sort of successful transplant to be achieved. Based on available data examining cellular viability via fluorescent staining, the average viability of Cartiform was established to be 70.5 percent. This is an average with a range established between 54.5 percent and 88.5 percent.[3] ProChondrix however, had an average viability of 87.5 percent (range 73.5 to 99) at its maximum shelf life of 35 days.

The cutoff for the viability needed for a successful transplant of cartilage allograft as established by Cook et al is shown in **Figure 4**.

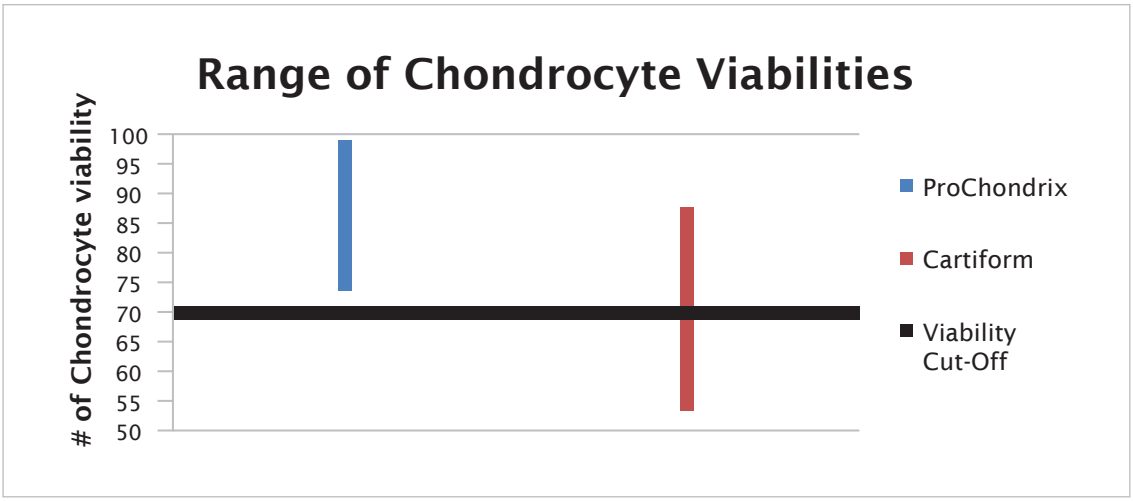

Figure 4. Range of chondrocyte viabilities for ProChondrix and Cartiform. The cutoff for the viability needed for a successful transplant as established by Cook et al is shown in black.

References

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