Evaluating the Effects of a Novel Storage Protocol for Long-Term Preservation of Fresh Osteochondral Allografts

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INTRODUCTION: Osteochondral allograft transplantation is a surgical technique involving the replacement of degraded cartilage with a size-matched graft harvested from a cadaveric donor that consists of viable hyaline cartilage and subchondral bone. These grafts have been reported to remain viable up to 10 years in 85% of patients, and over 20 years in over 65% of patients.[1] Success rates of transplantation increase when chondrocyte viability in the donor graft is maintained at a minimum of 70% viability compared to fresh control.[2-4] Storage in Lactated Ringer's solution (LRS) supplemented with antibiotics bacitracin and cefazolin maintains these levels for up to 14 days, and has been used by Mount Sinai Allograft Technologies (Toronto, Canada) and others.[1,3,4] Certain experimental and approved culture media-based storage solutions can maintain viability up to 28-56 days[4] but employ various proprietary formulations and storage temperatures that cannot be replicated.[2] The objective of this study was to formulate a novel storage solution that maximized chondrocyte viability and preserved cartilage properties up to 42 days, while still maintaining simplicity in the formulation. It was hypothesized that by storing donor tissues in a DMEM-based media, (1.0 g/L glucose, with L-glutamine, sodium pyruvate) supplemented with high molecular weight hyaluronic acid (HA) (1500-2000 kDa) and doxycycline (DOX), chondrocyte viability and histological appearance of the cartilage extracellular matrix would be maintained more effectively when compared to the LRS-based institutional standard.

METHODS: All procedures were performed with Institutional Animal Care Committee approval in accordance with the Canadian Council on Animal Care. The left and right distal femurs of 11 male New Zealand white rabbits (3.71 ± 0.4 kg), were harvested aseptically and stored in either LRS or supplemented DMEM solution, respectively, for 0 days (fresh control, n = 2), 7 days (n = 2), 21 days (n = 2), 28 days (n = 3) and 42 days (n = 2). Under both conditions, tissue was stored in 250 mL of media at 4°C, protected from light and subjected to weekly media changes. At each time point, cartilage was isolated from the medial and lateral femoral condyles and stained with Calcein AM (1.8 µM) and Ethidium Homodimer-1 (2.0 µM) to identify live and dead chondrocytes in the weight-bearing regions of the cartilage. Multiple cartilage pieces from each rabbit were imaged on a Leica SP8 confocal microscope at 10x objective (Figure 1) at day 0 (N = 7), day 7 (N = 4), day 21 (N = 7), day 28 (N = 12), and day 42 (N = 8). A custom-built program (MathWorks MATLAB R2022a) was used for analysis by segmenting and counting live and dead cells individually, as well as dual-stained cells classified as dead (Figure 2). Osteochondral explants from the distal trochlea were fixed in 10% formalin for 7 days, decalcified in 0.5 M ethylenediaminetetraacetic acid for approximately 7 days and embedded in paraffin wax. Sections, 5 µm thick, were stained with Safranin-O and Fast Green, and images were captured using an inverted brightfield microscope and evaluated qualitatively for retention of Safranin-O stain (Figure 3). Statistical analyses were performed using Welch's unequal variance t-test to compare the percent viable chondrocytes per storage condition and storage time (GraphPad Prism 10.0.2).

RESULTS: DMEM-stored tissues maintained chondrocyte viability to 42 days, while LRS-stored tissues exhibited a progressive increase in dead cells from the articular surface down toward the subchondral bone over time (Figure 1). When compared to LRS, absolute cell viability data (Figure 2A) revealed that chondrocyte viability was 19.6% higher ($p < 0.0001$) and 29.3% higher ($p = 0.0011$) after 28 and 42 days of storage in supplemented DMEM, respectively. When normalized to day 0 fresh control (Figure 2B), chondrocyte viability in LRS-stored samples was maintained above the 70% threshold at 83.3% and 74.1% at day 21 and 28, respectively, but decreased to 58.7% after 42 days. In contrast, chondrocyte viability in cartilage stored in supplemented DMEM was maintained above 90% up to the 42-day point when normalized to day 0 fresh control with viability measured to be 97.0% and 93.1% at 28- and 42-day time points, respectively (Figure 2B). No qualitative differences in Safranin-O retention were observed between histological sections taken from the trochlea of samples stored for 7, 28 and 42 days in both LRS and supplemented DMEM conditions (Figure 3).

DISCUSSION: Chondrocyte viability was maintained above 70% of fresh control in DMEM supplemented with HA and DOX for up to 42 days in storage. These results suggest the ability of HA to provide protection and lubrication within the natural joint environment[5] and the anti-apoptotic effects of DOX reported in tissue culture[6] contributed to the increased viability observed when compared to LRS storage. We hypothesize that HA provided a protective layer at the articular surface that prevented the depth-dependent pattern of cell death observed in LRS-stored samples. Histological staining indicated that both storage methods maintained proteoglycan content up to 42 days and may be due to rabbits retaining higher intrinsic repair abilities at maturity,[7] pointing to a need for further testing of the supplemented DMEM formulation on human or other animal tissues. A small sample size limited this study, however, we noted that variation among DMEM stored samples remained much lower at all time points when compared to LRS (Figure 2). High variability was observed among day 0 fresh controls due to an increased dead cell count in one sample analyzed 24 hours after sacrifice, which contributed to the relatively high normalized data at the day 7 time point. This study demonstrated the potential of the novel formulation to extend the time when tissues remain viable for transplantation.

SIGNIFICANCE: DMEM supplemented with HA and DOX maintained chondrocyte viability above 90% of fresh controls for up to 42 days in rabbit tissues. These data merit further exploration of this novel storage protocol to support efforts to increase the quality and quantity of fresh tissues for clinical use.

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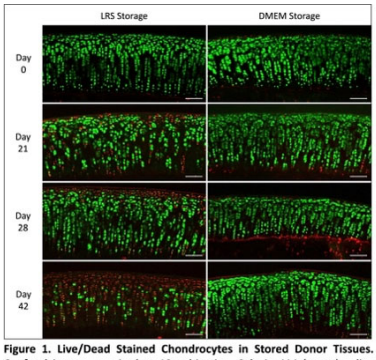

Figure 1. Live/Dead Stained Chondrocytes in Stored Donor Tissues. Confocal images acquired at 10x objective. Calcein AM (green) = live cells, Ethidium Homodimer-1 (red) = dead cells. Scale bar = 100 µm.

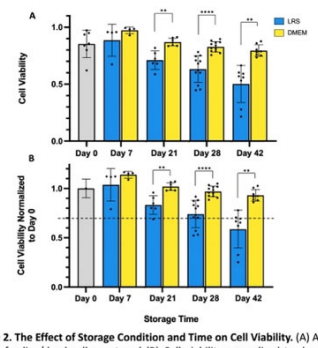

Figure 2. The Effect of Storage Condition and Time on Cell Viability. (A) Absolute values for live/dead cell count and (B) Cell viability normalized to day 0 fresh control. The dotted line represents the minimum 70% viability required for transplantation. ** = $p < 0.01$, **** = $p < 0.0001$, Welch's unequal variance t-test

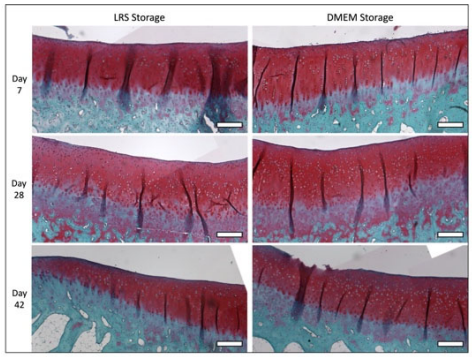

Figure 3. Safranin-O and Fast Green-Stained Trochlea Sections. Rabbit trochlea sections acquired at 4x objective after 7-, 28- and 42-day storage. Scale bar = 500 µm.