Long-Term Storage and Preservation of Tissue Engineered Articular Cartilage

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Disclosures:  A.B. Nover: None. S.L. Lee: None. W.T. Yu: None. R.M. Stefani: None. G.A. Ateshian: None. A.M. Stoker: None. J.L. Cook: 1; Arthrex. 3B; Arthrex. 3C; Schwartz Biomedical. 5; Arthrex, Zimmer, Synthes. C.T. Hung: None.

Introduction: Articular Cartilage focal defects are generally treated with osteochondral allografts (OCAs). Fresh OCAs have been shown to offer a long-term solution [1]. Now with mandatory disease/contamination screening which takes about 14 days, grafts are now stored prior to transplantation [2]. With the success of OCAs tied to the viability of the graft’s chondrocytes, which decreases over storage time [1, 3-4], surgeons see ~28 days as a maximum implantation window before grafts are considered “expired” [2]. Clinical allograft storage media typically contains serum and requires refrigeration [5]. Recently, Stoker et al. developed a storage protocol with the advantages of a serum-free media and room temperature that was able to maintain day 0 viability for 63 days (Missouri Osteochondral Allograft Preservation System, MOPS) [6-7]. While increasing preservation time will increase the impact of OCAs in the clinic, the demand for suitable allografts greatly outweighs their supply. As such, allogeneic, mechanically functional tissue engineered cartilage grafts can have the inherent benefits of native osteochondral allografts (OCAs) but without the disadvantages of limited supply, size matching, or wait time. In order to effectively translate these grafts into the clinic, there exists a need for a storage protocol for them as well, which may not be the same as for native tissue. Here, MOPS is applied to tissue engineered cartilage constructs and compared to continued culture as well as clinical storage media.

Methods: Study 1: Chondrocytes were isolated from adult canine knees, passaged once, and then cast into agarose disks (30 million cells/mL, 2% w/v agarose, 4 mm diameter, 2.3 mm thick). Constructs were cultured in chemically-defined media with added TGF-β3 (media changed 3X weekly) until reaching maturity (day 35 or 42). Then, a subset of samples were transferred to MOPS (no growth factor, media changed 1X weekly) while the remainder continued to be cultured. The continuous culture group is used for reference control, yet does not represent a clinically relevant scenario. Constructs were evaluated for mechanical, biochemical (DNA: PicoGreen dsDNA kit; glycosaminoglycan (GAG): 1,9-dimethylmethylene blue dye-binding assay; and collagen: orthohydroxyproline colorimetric assay), histological (GAG: Alcian Blue; and collagen: Picrosirius Red), metabolic (MTT), and viability (Live/Dead kit) properties for an additional 56 days. Media was also analyzed for biomarkers. This study was repeated, yielding similar results. Study 2: P1 canine chondrocytes were cast as previously described at both 30 and 60 million cells/mL. Following similar culture to maturity (day 42), a subset of constructs were transferred to MOPS (media changed 1X weekly) while the remainder were transferred to a clinical preservation system (media changed 3X weekly) [5]. Constructs were evaluated for mechanical, biochemical, histological, and viability properties 28 days later. Studies schematically represented in Fig. 1A. For both studies a two-way ANOVA with Tukey’s HSD post-hoc (α<0.05) was used to determine
significant differences between groups and the maturity point (start of preservation protocol) or each other.

**Results:** Study 1: At 28 days following maturity, live/dead stains of MOPS and control constructs were not ostensibly different from each other or from the maturity point. By 56 days of preservation, there was slightly more dead cells in both groups, but cell death remained minimal. Likewise, DNA content remained similar across groups and compared to the maturity point for the full 56 days (not shown). Equilibrium modulus (EY), dynamic compressive modulus (G*, not shown), and GAG content of preserved constructs remained similar to the maturity point for four weeks, yet decreased by eight weeks (Fig. 1B.). Collagen content was maintained for the full 56 days preserved (Fig. 1B.). There were no ostensible differences in histology across groups or compared to maturity point (not shown). MTT showed metabolism slowing over storage time (Fig. 1B.). Media analysis showed MMP-1, MMP-2, and MCP-1 content decreasing over time, while MMP-3 content elevates toward the end of preservation (not shown). Study 2: At 28 days preserved, constructs of both cell densities stored with the clinical protocol showed significantly higher EY, G*, and GAG per wet weight (WW) than those stored with MOPS (Fig. 1C.). Visually, media pH decreased in the MOPS group following transfer to storage media, though this was not seen in the clinical group. Collagen content was similar between both groups (not shown). Histology showed no apparent differences across groups (not shown).

**Discussion:** Looking ahead to the translation of tissue engineered articular cartilage grafts into the clinic, a preservation/storage protocol will be necessary to support their distribution and adoption. As a starting point, the MOPS protocol for allografts was applied to tissue engineered constructs (Study 1). This protocol offers the advantages of being chemically defined and easy to use (1X weekly media change, no refrigeration or incubation). The current findings are encouraging in that MOPS can preserve tissue properties of mature cartilage engineered from adult cells for approximately 28 days with some properties maintaining for even longer without the addition of growth factor, a necessity for growth of adult chondrocytes [8-9]. For longer storage periods, however, the more standard preservation culture yielded better maintenance of tissue properties (Study 2). While viability is not greatly impaired, cell metabolism slows over time (MTT, media analysis). The elevation of MMP-3 is consistent with native allograft storage seen in [10]. Differences in pH between groups may indicate a slowing of metabolism in the clinical storage group compared to MOPS. As multiple parameters vary between the examined protocols, future studies will investigate the effects of these differences, particularly that of temperature, which may be a key influencer of metabolism. The contrasting findings for the long-term (>28 day) efficacy of MOPS for preserving native OCAs (in our past study [6-7]) and in the current study of engineered OCAs may stem from intrinsic differences between native grafts and those that are fabricated de novo, such as metabolism. Given the advantages of MOPS over standard preservation protocols, namely serum-free and room temperature application, future efforts will aim to optimize its application for engineered cartilage grafts.

**Significance:** The development of a robust protocol for preservation/storage is critical for proper translation of tissue engineered articular cartilage grafts into the clinic.
Figure 1. A. Schematic of Studies: Study 1 compares MOPS to continuous culture; Study 2 compares MOPS to protocol more representative of current clinical storage conditions. B. Study 1: Equilibrium Modulus, GAG/WW, Collagen/WW, MTT Absorbance (Normalized By Absorbance at Maturation Point), cross-sectional Live/Dead Imaging of constructs placed in Continuous Culture Control (blue) and MOPS (orange) at day 28 and 56 past maturity point (dashed line). * indicates statistically significant difference between control and MOPS. C. Equilibrium Modulus, GAG/WW at different cell densities (30 and 60 Million/mL).