Frequent Chondroitinase Treatment in Engineered Cartilage with Native Level of Cell Seeding Density Does Not Enhance Collagen Deposition and is Detrimental to Chondrocytes

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INTRODUCTION: Articular cartilage is an avascular, load-bearing tissue with a dense extracellular matrix (ECM) synthesized by chondrocytes (~10^8 cells/ml in immature human tissue) [1]. The predominant ECM constituents, proteoglycans and collagen, allow cartilage to support the high compressive and tensile loads experienced in diarthrodial joints. The goal of tissue engineering is to recreate a functional ECM such that constructs implanted into osteoarthritic defects can withstand *in vivo* loads. Previous work has successfully replicated the glycosaminoglycan (GAG) content and compressive modulus of native tissue in engineered cartilage with temporary growth factor supplementation [2,3]. Achieving native levels of collagen remains a challenge in the development of a functional ECM. One promising strategy targeting native collagen composition is treatment with chondroitinase ABC (CABC), an enzyme which degrades both chondroitin and dermatan sulfates without disrupting the collagen network [4-7]. Previous work in the [primary bovine chondrocyte] – [agarose gel] tissue engineering system has shown collagen density and tensile mechanical properties are increased in constructs with a cell seeding density of 30×10^6 cells/ml when treated infrequently with CABC [5]. Based on this promising result this study aims to promote further ECM and collagen deposition through the use of frequent CABC supplementation, using a cell seeding density that better reflects the cellularity of immature articular cartilage.

METHODS:CULTURE: Bovine chondrocytes were harvested and encapsulated in a 2% agarose gel scaffold (Type VII, Sigma) at a concentration of 120×10^6 cells/mL [2]. Cylindrical constructs (∅3 mm × 2.3 mm) were cultured in chemically-defined, chondrogenic media supplemented with 10 ng/ml TGF-β3 for the first 14 days of culture [2]. CABC TREATMENT: Starting on day 14, constructs were treated with 0.15 U/ml CABC (Sigma) for two consecutive days per week. Constructs received treatment for either one week (group CABC1; week 3), four weeks (group CABC2; weeks 3-6), or seven weeks (group CABC3; weeks 3-9). A control received no treatment. MECHANICAL PROPERTIES: Sample thickness and diameter were measured before mechanical testing. Equilibrium compressive modulus (E_Y) was measured by first equilibrating constructs under a creep load for 300s before prescribing 10% strain (ramped over 200s) and relaxing for 1600s. E_Y was computed from the resulting equilibrium load. BIOCHEMISTRY: Constructs were digested with proteinase K and assayed for GAG (DMMB assay), collagen (orthohydroxyproline assay), and DNA (PicoGreen DNA assay). STATISTICS: Properties were analyzed with a two-way ANOVA and post-hoc analysis (Tukey HSD test; α=0.05; n=4 per group per time point). Only final time point (day 77) results are reported here.

RESULTS: Swelling ratio was significantly affected by CABC treatment ($p<0.001$, fig. 1A), with a seven-fold increase observed for the control and a three-fold increase for CABC1; no significant swelling was observed for CABC2 and CABC3 ($p=0.995$). Due to such large changes in construct volume, GAG and collagen content results vary dramatically when normalized to day 0 (D0) or day 77 (D77) construct wet weight (ww); both representations are reported here. When normalized to D0 ww, GAG and collagen content decreased significantly with CABC treatment (GAG: $p<0.001$, fig. 2A; collagen: $p<0.05$, fig. 3A). When normalized to D77 ww, GAG content was highest in control and CABC1 groups ($p<0.001$, fig. 2B); collagen content was also influenced by CABC treatment ($p<0.001$, fig. 3B). E_Y, evaluated using D77 construct dimensions, was highest in the CABC1 groups ($p<0.05$, fig. 1B). Cells (DNA) per construct decreased significantly with increasing CABC treatment up to 4 treatments ($p<0.005$, fig.1C).

DISCUSSION: Previous studies have suggested that excessive GAG deposition at early time points may inhibit collagen deposition by "crowding it out." Therefore, in this study, CABC was administered to constructs using multiple treatments, in an attempt to increase collagen content to native levels; to facilitate this process, a cell seeding density comparable to native levels was used. When using D77 dimensions and ww for normalization, this study confirms earlier findings that CABC treatments increase collagen content (fig. 3B) [4-6]. However, decreasing DNA content with increasing CABC treatments (fig. 1C) indicates that CABC is not harmless to chondrocytes, raising doubts about the benefits of this strategy for cartilage tissue engineering. When ECM composition is normalized to D0 ww, it becomes evident that the best results (highest amount of total matrix deposition) are achieved with the untreated control group (figs. 2A & 3A). The only functional measure that is seemingly less favorable in the control versus the CABC1 group is E_Y. This is consistent with the understanding that the E_Y of cartilage increases with increasing proteoglycan fixed charge density, which is proportional to GAG content normalized to final wet weight. Hence, the trends for E_Y in fig. 1B are similar with the trends in GAG content in fig. 2B. Interestingly, the high cell seeding density utilized here (120×10^6 cells/mL), comparable to native levels, produces growth in the control, with no loss of cell content from lack of sufficient nutrient supply (fig.1C). The seven-fold swelling ratio observed in this study (fig. 1A) exceeds the two-fold ratio observed in our earlier study seeded at 30×10^6 cells/mL [5].

SIGNIFICANCE: Chondroitinase treatment of cartilage constructs may be harmful to chondrocytes, producing lower total amounts of matrix deposition, suggesting this strategy may not be as beneficial for enhancing total collagen deposition as previously thought. Elevated cell seeding densities may attenuate such affects and lead to more favorable outcomes. Future improvements in E_Y, GAG, and collagen content normalized to final construct weight might be achieved by suitably constraining the swelling of constructs with high cell seeding during the growth process.

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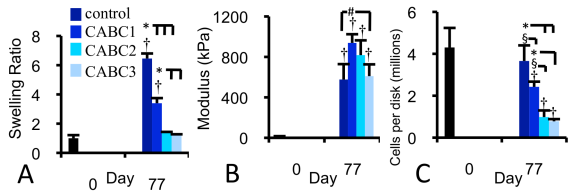

Figure 1: Swelling ratio (a), compressive modulus (b), and cell count (c) of D0 and D77 constructs. † represents change from day 0 mass. ($p<0.05$); within time points * denotes $p<0.0005$, § denotes $p<0.005$, # denotes $p<0.05$.

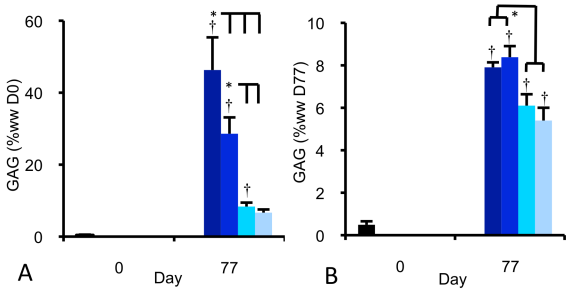

Figure 2: GAG composition normalized to (a) D0 mass; (b) D77 mass.

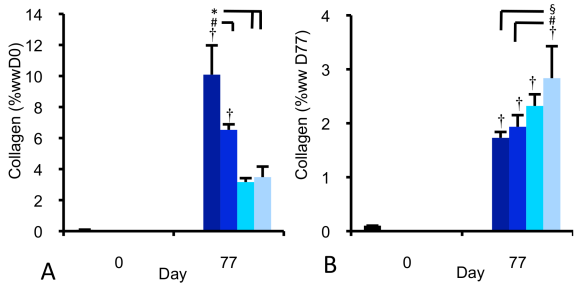

Figure 3: Collagen composition normalized to (a) D0 mass; (b) D77 mass.