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PROLONGED TREATMENT OF ULTRA-LOW DOSE CHONDROITINASE ABC IMPROVES MATRIX PRODUCTION IN ENGINEERED CARTILAGE

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INTRODUCTION

Articular cartilage is the load bearing soft tissue of diarthrodial joints, and mechanical loading maintains the integrity of the tissue. The predominant extracellular matrix constituents, proteoglycans and collagen, allow cartilage to support the high compressive and tensile loads experienced in diurnal loading. Our laboratory has been successful in cultivating engineered cartilage constructs with a compressive equilibrium modulus and glycosaminglycan (GAG) content near native values [1, 2]. Many approaches to cultivating engineered cartilage have been limited by low collagen production *in vitro*, an impediment for attaining native functional load-bearing properties [3].

Previously we, and others, have demonstrated that digestion of mature constructs with a high dose (≥0.15 U/mL) of chondroitinase ABC (chABC) temporarily suppresses the GAG content, increases collagen content and eventually improves mechanical properties [2, 4-5]. Compressive mechanical properties and GAG content of chABC-digested constructs are restored to the undigested control values within 4-6 weeks of culture. However, the optimal dose and duration of chABC treatment for improving the biochemical and mechanical properties of engineered cartilage is not well understood.

Recently, we evaluated the effect of repeated treatments of chABC as a way to further increase the collagen content in our engineered constructs. However, repeated digestion with a high-dose of chABC (0.15U/mL for 2 days) caused extensive cell death and was detrimental to the long-term mechanical and biochemical properties of engineered cartilage [6]. It is possible that a low dose of chABC can be used to find an optimal balance between enhanced properties and cell death.

Therefore, the objective of this study was to evaluate the effect of media supplementation with a low dose of chABC during one week in culture compared to a short-term treatment (2 days) of a higher dose of chABC. We hypothesize that the lower dose over a longer period will be as effective as the higher dose given during 2 days in culture.

METHODS AND MATERIALS

Chondrocytes were harvested from juvenile bovine knee joints and passaged in DMEM supplemented with 10% serum, 1% 100 U/ml penicillin, 100 mg/ml streptomycin and amphotericin B (Invitrogen Co., Carlsbad, CA), 1 ng/ml TGF-β1, 10 ng/ml PDGF-ββ, and 5 ng/ml bFGF2 [1]. Passaged cells were seeded in a slab of 2% w/v agarose with a cell concentration of 30 x 10^6 cells/mL. Constructs were cored from the slab (∅4 mm × 2.34 mm thick) and cultured in chemically-defined media supplemented with 10 ng/mL of TGF-β3 for the first 14 days. At day 14, constructs were divided into four groups, with three of the groups receiving thermo-stabilized chABC [7]. Thermo-stabilized chABC was used to maintain chondroitinase activity at 37°C between media changes. As described previously, the culture medium was supplemented with 0.15 U/mL of chABC (high-dose group) [2]. Two other groups received a lower dose of chABC, which represented 2.5% and 10% of the high-dose group (Fig 1). That is, the culture medium was supplemented with 0.004 U/mL or 0.015 U/mL chABC between days 14 and 21. Remaining (undigested) constructs served as control.

At day 14 and 56, mechanical and biochemical properties were assessed (n = 5-7 per group). The equilibrium Young's modulus (E_Y) was determined using unconfined compression stress-relaxation at 10% strain. The dynamic modulus was measured from the response to a superposed sinusoidal input of

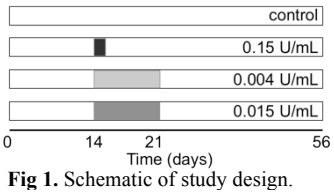

Fig 1. Schematic of study design.

±1% strain at 0.5 Hz. DNA content was determined using the PicoGreen Kit (Invitrogen Co). GAG and collagen content were determined using the 1,9-dimethylmethylene blue (DMMB) and hydroxyproline assays, respectively. A one-way ANOVA was performed on mechanical and biochemical properties with a Bonferroni post-hoc. Significance was set at $p \leq 0.05$.

RESULTS

At day 56, the diameter and thickness of control constructs were greater than the dimensions of chABC treated constructs ($p < 0.01$; Fig 2). The final thickness of the 0.004 U/mL group was 2.72 ± 0.10 mm and was 10% lower than the control group ($p < 0.01$). The Young's modulus of the control group was 603 ± 62 kPa (Fig 3A). By day 56, only the 0.004 U/mL group had a compressive Young's modulus comparable to the undigested control (Fig 3A). The dynamic modulus of the 0.15 U/mL and 0.004 U/mL groups was 25-30% greater than the control ($p < 0.02$; Fig 3B). By day 56, the Young's modulus of the 0.015 U/mL group was approximately 50% of the day 14 values (Fig 3A).

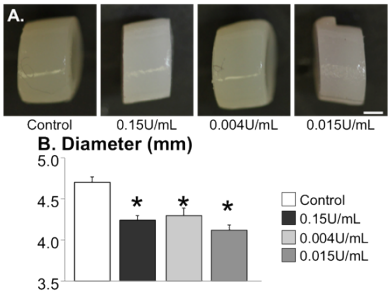

Fig 2. (A) Representative images and (B) final diameter for each group. *p < 0.05 vs control. Bar = 1mm.

The DNA content for chABC-digested constructs was lower at day 56 than the control group at day 14, with the 0.15 U/mL group having the largest decrease in DNA content (Fig 4A). The GAG content normalized by DNA for the 0.004 U/mL and the 0.15 U/mL group was more than 2X greater than the control (Fig 4B). The GAG content normalized by wet weight was 9.5 ± 0.7 %/ww for the 0.004 U/mL group and was 20% higher than the control (7.8 ± 1.5 %/ww, $p = 0.04$). The GAG content normalized by wet weight for the 0. 15 U/mL group was not significantly different from the control group.

By day 56, the 0.004 U/mL group had the highest collagen content (3.25 ± 0.37 %/ww) and was 65% higher than the control ($p < 0.001$; Fig 4C). The collagen content of the 0.004 U/mL and the 0.15 U/mL groups was significantly greater than the control for all normalization methods (i.e. normalization by DNA, wet weight or dry weight; $p < 0.02$; Fig 4C & D).

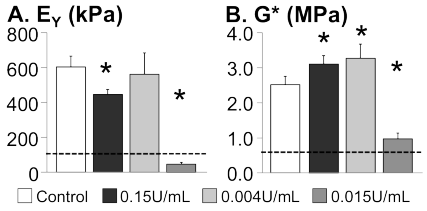

Fig 3. (A) Compressive Young's modulus (E_Y) and (B) dynamic modulus at day 56. *p < 0.05 vs. control. Dashed line represents day 14 values.

DISCUSSION

Improving collagen production in engineered cartilage is important for recapitulating the high tensile stiffness of native cartilage, which is crucial for preventing excess lateral expansion under compression [9]. While chABC treatment can be beneficial for improving collagen production in engineered cartilage [2, 4], it is detrimental to cell viability. We observed a significant drop in DNA content that was sustained throughout the culture period (Fig 4A) [6]. Therefore, the concentration and frequency of treatment must be optimized. In this study, we evaluated the effect of a low-dose chABC treatment during one week in culture and compared it to a high dose treatment that has been used previously [2, 3, 6, 8].

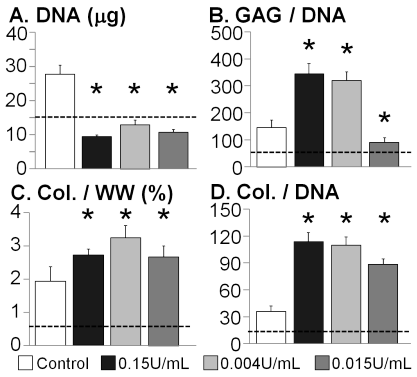

Fig 4. (A) DNA content, (B) GAG content normalized by DNA, (C) collagen content normalized by wet weight (ww), and (D) collagen content normalized by DNA. * $p < 0.05$ vs. control. Dashed line represents day 14 values.

The ultra-low chABC treatment group (0.004 U/mL) demonstrated an ability to fully recover compressive mechanical integrity, while improving the overall biochemical composition (Fig 3 & 4). Furthermore, the 0.004 U/mL group had a higher dynamic modulus than the control group, which has been correlated with an increase in the tensile modulus [8]. This is likely due to the increased collagen content or an increase in the collagen fibril diameter following chABC treatment [5]. Importantly, the collagen content of the ultra-low dose chABC group was 65% greater than the control group, compared to a 40% increase in collagen content with the high-dose of chABC (Fig 4C). These findings suggest that an ultra-low chABC treatment may be more advantageous for improving the compressive modulus 'recovery time' as well as increasing the overall biochemical composition and tensile properties of engineered cartilage.

Biochemical composition and mechanical properties are equally important characteristics of engineered cartilage. The 0.015 U/mL group had a higher collagen content than the control group. However, the compressive modulus did not return to day 14 values after the five-week recovery period following the chABC insult (Fig 3). Taken together, these findings suggest that long-duration treatment of chABC at concentrations greater than 0.004 U/mL may prevent cell proliferation and matrix production.

The total chABC delivered to the low-dose chABC groups was much lower than the high dose concentration, which affected the drop in DNA content observed (Fig 4A). The drop in DNA content was not correlated to recovery of the mechanical integrity of engineered cartilage (Fig 3A). These findings suggest that long-duration treatment may have a greater effect on cell production than cell viability.

We achieved a 65% increase in the collagen normalized by wet weight with the ultra-low dose of chABC (3.3%/ww at day 56). However, it should be noted that the collagen content of native cartilage is 10-15% by wet weight [9, 10]. Therefore, we were able to cultivated engineered cartilage with collagen composition that approximately 30% of native values, demonstrating the challenge of recapitulating collagen composition and orientation in engineered cartilage. In conclusion, the results of this study demonstrate that longer chABC treatment (one week) at ultra-low concentrations can be used to further enhance collagen production and decrease the culture time needed to recover the construct's mechanical integrity.

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