TIME AND DOSE-DEPENDENT EFFECTS OF CHONDROITINASE ABC ON GROWTH OF ENGINEERED CARTILAGE

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Abstract

Tissue engineering techniques have been effective in developing cartilage-like tissues *in vitro*. However, many scaffold-based approaches to cultivating engineered cartilage have been limited by low collagen production, an impediment for attaining native functional load-bearing tensile mechanical properties. Enzymatic digestion of glycosaminoglycans (GAG) with chondroitinase ABC (chABC) temporarily suppresses the construct's GAG content and compressive modulus and increases collagen content. Based on the promising results of these early studies, the aim of this study was to further promote collagen deposition through more frequent chABC treatments. Weekly dosing of chABC at a concentration of 0.15 U/mL resulted in a significant cell death, which impacted the ability of the engineered cartilage to fully recover GAG and compressive mechanical properties. In light of these findings, the influence of lower chABC dosage on engineered tissue (0.004 and 0.015 U/mL) over a longer duration (one week) was investigated. Treatment with 0.004 U/mL reduced cell death, decreased the recovery time needed to achieve native compressive mechanical properties and GAG content, and resulted in a collagen content that was 65 % greater than the control. In conclusion, the results of this study demonstrate that longer chABC treatment (one week) at low concentrations can be used to improve collagen content in developing engineered cartilage more expediently than standard chABC treatments of higher chABC doses administered over brief durations.

Keywords: Cartilage tissue engineering, chondroitinase ABC, collagen, glycosaminoglycans, articular cartilage, enzymatic digestion, dose dependence.

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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 (ECM) constituents, proteoglycans and collagen, allow cartilage to support the high compressive and tensile loads experienced during diurnal loading. Tissue engineering techniques have been effective in developing cartilage-like tissues *in vitro* (Asanbaeva *et al.*, 2008; Bian *et al.*, 2009b; Huang *et al.*, 2010; Johnstone *et al.*, 2013; Lima *et al.*, 2007; Moutos and Guilak, 2010; Nguyen *et al.*, 2012; O'Connell *et al.*, 2012; Sampat *et al.*, 2011). Our prior studies have been successful in cultivating engineered cartilage constructs that have compressive mechanical properties and glycosaminoglycan (GAG) content near native values (Bian *et al.*, 2009a; Lima *et al.*, 2007; Sampat *et al.*, 2011). However, these studies as well as the majority of those in the literature which feature cells embedded in a scaffold have been limited by low collagen production, an impediment for attaining native functional load-bearing tensile mechanical properties (O'Connell *et al.*, 2012; Riesle *et al.*, 1998). Interestingly, the collagen content within scaffoldless culture systems tends to exceed that of systems with a scaffold, while the GAG content of such systems tends to be lower than native cartilage (Huey *et al.*, 2012).

The remarkable degree of resilience and deformability in native cartilage is due to the high density of both collagen (10-20 %/ww) and proteoglycans (4-6 %/ww) (Klein *et al.*, 2007). Under physiological loading, cartilage experiences both high compressive strains (10-15 %) and tensile strains (~6 %) in the superficial layer, which results as the two articulating surfaces contact one another (Canal *et al.*, 2008; Chahine *et al.*, 2004; Guterl *et al.*, 2009; Miller and Morgan, 2010; Zhang *et al.*, 2005). In native cartilage, these large tensile strains are supported predominantly through the tissue's dense fibrillar collagen network. Presently, engineered cartilage, both scaffold and scaffoldless systems, lack the tensile robustness of native tissue (approximately 20 MPa) (Park and Ateshian, 2006). Increasing the collagen content of engineered cartilage has been correlated with improvements in tensile mechanical properties (Kelly *et al.*, 2013; Natoli *et al.*, 2009b). Therefore, it is likely that ensuring engineered tissues can withstand the physiologic loading conditions will require recapitulating the native tissue's collagen architecture and composition (Adeeb *et al.*, 2004; Canal *et al.*, 2008; Chahine *et al.*, 2004; Guterl *et al.*, 2009).

Previously we, and others, have demonstrated that enzymatic digestion of mature engineered cartilage

constructs can be used to improve relative collagen production (Bian *et al.*, 2009b; Natoli *et al.*, 2009a; Natoli *et al.*, 2009b; Ng *et al.*, 2009; Responte *et al.*, 2012). Chondroitinase ABC (chABC) is more typically used to promote cartilage graft-host tissue integration (e.g., Quinn and Hunziker, 2002), through enhancing cell attachment to extracellular matrix. It should be noted that recruitment of synovium-derived stem cells to a chondral defect site is improved by treating the joint with a high dose of chABC (0.1 U/mL) *in situ* (Lee *et al.*, 2000). chABC cleaves chondroitin sulphate, dermatan sulphate, and hyaluronic acid, affecting proteoglycans such as aggrecan and decorin. As the cleaved proteoglycans diffuse from engineered cartilage constructs and are lost into the media, there is a temporary suppression of the construct's GAG content and compressive modulus as well as a concomitant increase in collagen content (Bian *et al.*, 2009b; Natoli *et al.*, 2009a; Natoli *et al.*, 2009b; Ng *et al.*, 2009; Responte *et al.*, 2012). Asanbaeva *et al.* (2008) demonstrated a threefold increase in tensile mechanical properties of cartilage constructs following chABC treatment. Furthermore, collagen fibres are thicker following enzymatic digestion, which may suggest that the deposited collagen after digestion undergoes maturation and fibril growth with less hindrance than the collagen in undigested constructs (Natoli *et al.*, 2009a).

Although most uses of chABC treatment on engineered cartilage have been limited to single dose treatments, previous work has suggested that two treatments can be used to further increase collagen content and tensile properties (Bian *et al.*, 2009b; Natoli *et al.*, 2009a). However, these studies applied a second treatment after a two- to four-week recovery period following the first chABC treatment, significantly extending the culture time needed to develop mature engineered cartilage. Full recovery of GAG and compressive mechanics can require up to an additional six weeks in culture (Bian *et al.*, 2009b). Based on the promising results of these early studies with chABC, this study aims to promote further extracellular matrix and collagen deposition through more frequent chABC treatments.

Our prior studies utilised a cell-based strategy to engineer articular cartilage by encapsulating cells in a clinically-relevant agarose hydrogel (Selmi *et al.*, 2008). An ideal biological replacement strategy would use a biodegradable scaffold; however, agarose is a non-biodegradable material. Degradation of agarose hydrogel with agarase has demonstrated temporary suppression of compressive mechanical properties and GAG content with an associated increase in collagen content, similar to the effects observed with chABC treatment (Bian *et al.*, 2009b). However, for this study, the aim was to better understand dosage- and time-dependent effects of chABC on engineered cartilage development, as this may provide a more generally applicable strategy for increasing collagen content in both scaffold and scaffold-free culture systems.

The optimal dose and duration of chABC treatment for improving the biochemical and mechanical properties of engineered cartilage are not well understood. Our previous work used a chABC concentration of 0.15 U/mL applied for 48 h, digesting approximately 85 % of the deposited

GAG in engineered constructs (Bian *et al.*, 2009b). The objective of this study was twofold: first, to evaluate the effects of one or four weekly 2-day doses of 0.15 U/mL chABC on engineered cartilage; second, to evaluate the effect of very low doses of chABC supplementation during a single longer culture period (7-day dose at 0.004 and 0.015 U/mL). The first hypothesis was that multiple 2-day doses would produce more collagen deposition than a single 2-day dose. The second hypothesis was that a 7-day dose at lower concentration would be less harmful to cells than a single 2-day dose at higher concentration, thereby producing improved matrix deposition and mechanical properties.

Materials and Methods

Cell harvest and construct culture
Chondrocytes were isolated from articular cartilage of bovine calf (3-6 week old) joints (carpometacarpal in study 1 and knee in study 2) digested with type IV collagenase in high glucose Dulbecco's Modified Eagle's Medium (hgDMEM) (Invitrogen Co., Carlsbad, CA, USA) supplemented with 5 % foetal bovine serum (Invitrogen), amino acids, buffering agents, and 1 % antibiotic-antimycotic (Invitrogen) at 37 °C on an orbital shaker. Primary (study 1) or passaged (study 2, defined below) cells were encapsulated in 2 % agarose (type VII-A; Sigma-Aldrich, St. Louis, MO, USA) at a density of 30×10^6 cells/mL and constructs were cored from the cell-agarose mixture (Ø4 mm × 2.3 mm thick) and cultured in chemically-defined, chondrogenic media (CM: hgDMEM with 0.1 μM dexamethasone, 50 μg/mL L-proline, 50 μg/mL ascorbate 2-phosphate, 100 μg/mL sodium pyruvate (Sigma-Aldrich), 1 × ITS+ premix (Becton Dickinson, Sparks, MD, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin and amphotericin B (Invitrogen)) at 37 °C under 5 % CO_2. Media were supplemented with 10 ng/mL transforming growth factor beta-3 (TGF-β3; R&D Systems, Minneapolis, MN, USA) for the first 14 d of culture (Lima *et al.*, 2007). Based on our prior work which suggested that chABC treatment can be deleterious for constructs when administered before day 14, chABC treatment was initiated on day 14, concurrently with TGF-β3 withdrawal (Bian *et al.*, 2009b).

Study 1 – Weekly supplementation of chABC
Study 1 examined the influence of a single or multiple weekly 2-day chABC treatments on the biochemical composition and mechanical properties of engineered cartilage. Starting on day 14, constructs were treated with 0.15 U/mL chABC (Sigma-Aldrich) for two consecutive days per week. Constructs (*n* = 4 per group) received chABC supplementation in the culture media either once (group CABC1; days 14-16) or four times (group CABC2; days 14-16, 21-23, 28-30 and 35-37; Fig. 1A). A corresponding control received no chABC treatment. Media were changed three times per week and constructs were cultured for 77 d.

Study 2 – Low-dose supplementation of chABC
To assess the transient influence of sustained chABC exposure to engineered cartilage, a preliminary study was

performed in which mature chondrocyte-seeded constructs (compressive Young's modulus $E_Y = 185.9 \pm 51.8$ kPa, $n = 3$; day 42 from casting) were cultured for 11 d without refreshing the media. Constructs were cultured with media supplemented with 0.15 U/mL of chABC on day 0. Control constructs were not supplemented with chABC. On days 1, 4, 8 and 11 samples were removed to measure GAG content using the 1,9-dimethylmethylene blue (DMMB) assay ($n = 5$ per group per time point) (Farndale *et al.*, 1986).

Study 2 examined the effects of very low doses of chABC delivered over a single 7-day culture period. Chondrocytes were harvested from juvenile bovine knee joints and passaged (P2) in hgDMEM supplemented with 10 % serum, 1 % 100 U/mL penicillin, 100 mg/mL streptomycin and amphotericin B, 1 ng/mL TGFβ1, 10 ng/mL platelet-derived growth factor (PDGF-ββ, from Invitrogen), and 5 ng/mL basic fibroblast growth factor (bFGF2) (Sampat *et al.*, 2011). Constructs were then cast using passaged chondrocytes. Study 2 was also repeated with primary chondrocytes and similar observations were observed; therefore, only the results from the passaged constructs are reported here.

At day 14, constructs were divided into four groups, with three groups receiving chABC. In one group, the culture medium was supplemented at the same dosage as in Study 1, 0.15 U/mL of chABC for two days (high-dose group) (Bian *et al.*, 2009b). Two other groups received a lower dose of chABC, which represented 2.5 % (0.004 U/mL) and 10 % (0.015 U/mL) of the high-dose group over a week-long supplementation period (Fig. 1B). Fresh chABC of these two groups was supplied during each media change between days 14 and 21 (3 media changes per week). Mechanical and biochemical properties were assessed at days 14 and 56 ($n = 5$-7 per group).

Mechanical and biochemical characterisation

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 ±1 % strain at 0.5 Hz. Prior to biochemical analysis, construct wet weight was measured. Swelling ratio (SR) was computed by normalising the current day's wet weight by the average wet weight on day 0 for each study. Samples were digested with proteinase K (MP Biomedical, Santa Ana, CA, USA) as previously described (Hollander *et al.*, 1994). DNA content was determined using the PicoGreen Kit (Invitrogen). GAG and collagen contents were determined using the DMMB and hydroxyproline assays, respectively (Farndale *et al.*, 1986; Hollander *et al.*, 1994). ANOVA were performed (Study 1: factors were day and treatment; Study 2: factor was treatment) on mechanical and biochemical properties. When significance was observed, a Bonferroni-corrected *post-hoc* test of the means was performed. Significance was set at $p \leq 0.05$. Data are presented as mean ± standard deviation.

Results

Study 1

Undigested control constructs developed E_Y, GAG concentration, and collagen concentration similar to
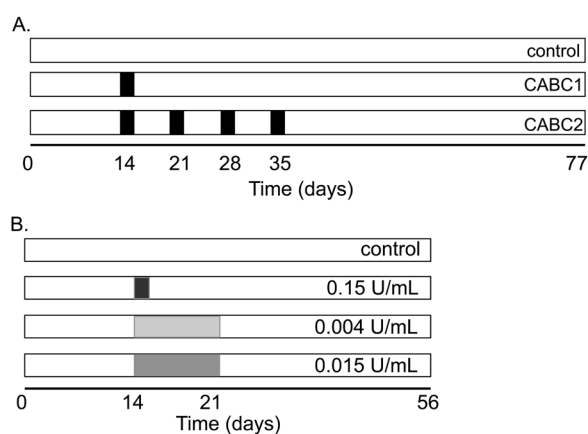

Fig. 1. Schematic of study design for (**A**) Study 1 and (**B**) Study 2. In Study 1, groups were differentiated by the number of weeks during which samples received chABC supplementation. In Study 2, groups were differentiated by the dosage of chABC, which was administered over a single week. Shaded regions represent the duration (time) and intensity (dosage) of chABC administration.

previous studies (Fig. 2A,C,D) (Byers *et al.*, 2008; Lima *et al.*, 2007). Both E_Y and dynamic modulus were diminished to near day 0 levels following chABC treatments on day 14 ($p < 0.05$; Fig. 2A,B, respectively); from these near zero levels, E_Y and dynamic modulus in the CABC1 group recovered to control levels by day 77 (Fig. 2A,B; $p = 0.98$). In contrast, E_Y and dynamic modulus in the CABC2 group failed to recover to control levels over the following 6 weeks. Similarly, GAG concentration (normalised to current day wet weight) was similar to control values in the CABC1 group (Fig. 2C; $p = 0.98$), but was different between the control and CABC2 group on day 77 ($p < 0.05$). Collagen levels (normalised to current day wet weight) were similar across all three groups for all time points (Fig. 2D; $p = 0.98$). After 77 d of culture, the cell content per construct was similar between the control and CABC1 group (Fig. 2E; $p = 0.52$), but the cell count per construct of the CABC2 group was significantly lower than the control ($p < 0.05$).

There was an observable and significant difference in the expansion and physical growth between the treatment groups of this study. Construct wet weight on day 0 was 24.4 ± 0.3 mg. By day 77, tissue swelling in the control group was significantly higher than both chABC-treated groups (Fig. 3A; SR ~ 3; $p < 0.001$). Furthermore, the SR of CABC1 (nearly twofold increase in wet weight) was significantly higher than that of CABC2 (SR ~ 1; $p < 0.001$). Due to these significant differences in construct wet weight, GAG and collagen contents were also analysed when normalised by the initial (day 0) construct wet weight (Fig. 3B,C). This normalisation provided a more accurate measure of the total ECM synthesis per construct. When the GAG content is normalised to the reference (day 0) configuration, one chABC treatment significantly decreased GAG content from the untreated
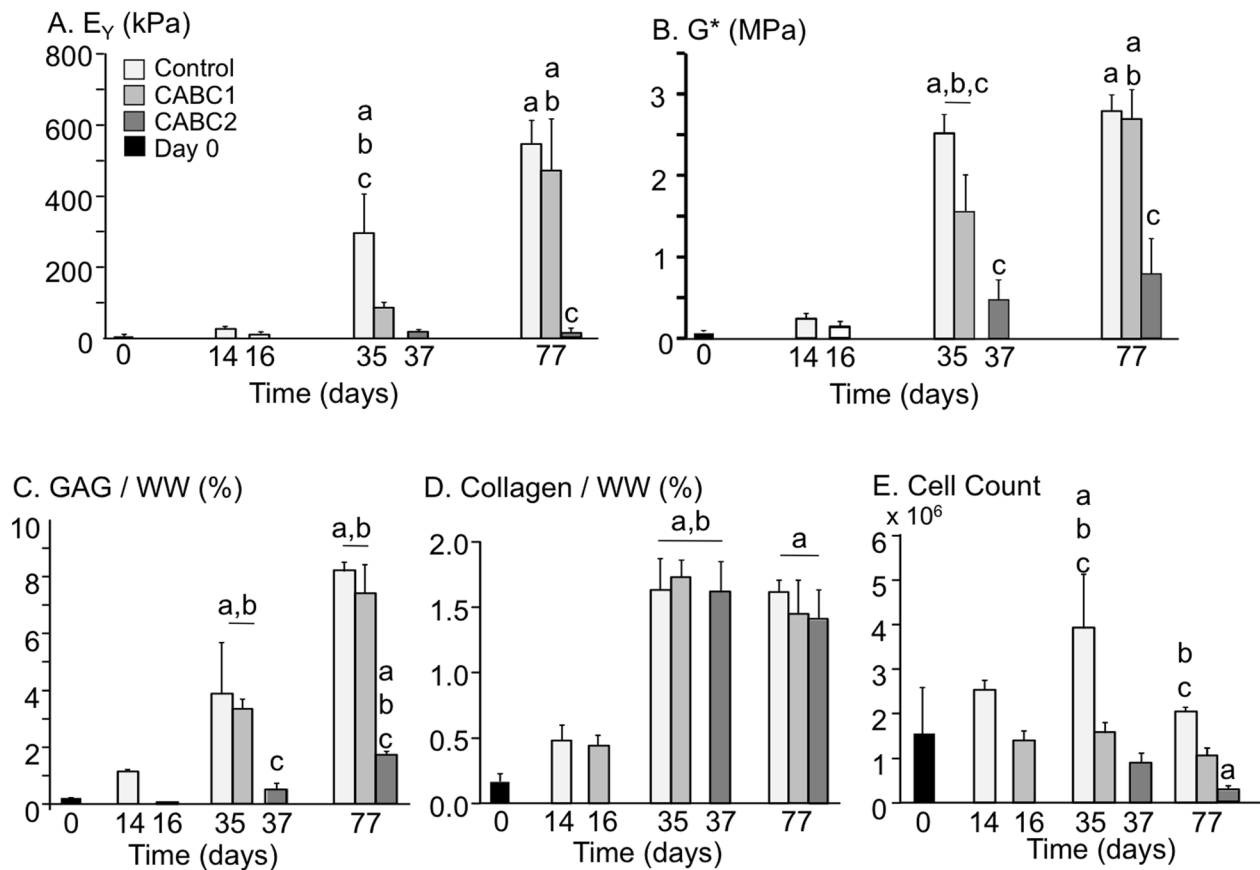
Fig. 2. Mechanical and biochemical properties of constructs from Study 1: (**A**) E_Y, (**B**) G^*, (**C**) GAG concentration relative to current day wet weight, (**D**) collagen concentration relative to current day wet weight, and (**E**) total number of cells within the construct. 'a' denotes difference from day 0 ($p < 0.05$); 'b' denotes difference from previous time point ($p < 0.05$); 'c' denotes difference from other groups at time point ($p < 0.05$).
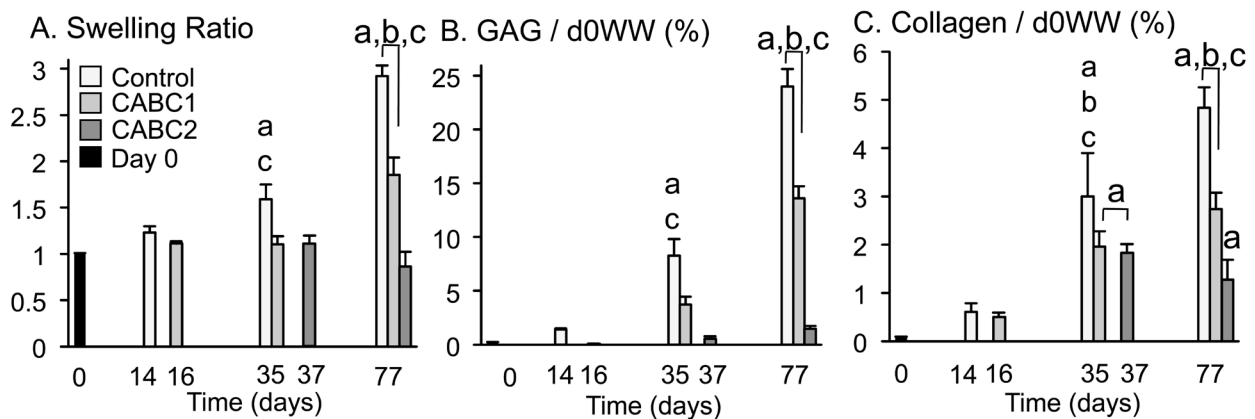

Fig. 3. The (**A**) swelling ratio, calculated as current wet-weight divided by day 0 wet weight, and biochemical properties (**B**) GAG and (**C**) collagen normalised to the construct wet weight at day 0. The day 0 wet weight for study 1 was 24.4 ± 0.3 mg. 'a' denotes difference from day 0 ($p < 0.05$); 'b' denotes difference from previous time point ($p < 0.05$); 'c' denotes difference from other groups at time point ($p < 0.05$).

control (Fig. 3B; $p < 0.001$); there was a further decline in total construct GAG with increasing the number of chABC treatments (Fig. 3B; $p < 0.001$). Similarly, total collagen content decreased with multiple chABC treatments (Fig. 3C; $p < 0.001$).

Study 2
Preliminary work for Study 2 investigated the effect of sustained chABC activity following a single treatment application (0.15 U/mL). The GAG content of chABC-digested constructs continued to decrease through day 8 (Fig. 4), at which point, chABC activity decreased, which

is comparable to previously reported chABC activity (Lee *et al.*, 2010). The GAG content for the control constructs did not change over the 11-day culture period, which is consistent with GAG data previously reported for mature engineered cartilage constructs (Bian *et al.*, 2009b). For Study 2, low-dose chABC treatments were applied fresh during each media change between days 14 and 21. The results from the preliminary study confirm chABC enzyme activity between media changes.

At day 56, gross images of the engineered cartilage demonstrate differences in final dimensions (Fig. 5A). The diameter and thickness of control constructs were greater than the dimensions of chABC-treated constructs ($p < 0.01$). The final thickness of the 0.004 U/mL group was 2.72 ± 0.10 mm, significantly lower than the control group ($p < 0.01$). Similar to the results in Study 1, the swelling ratio of chABC-treated constructs was significantly lower than the control ($p < 0.001$; Fig. 5B). Construct wet weight of undigested control constructs on day 56 was 52.02 ± 1.46 mg and was approximately twofold higher than the wet weight on day 0 of 26.6 ± 0.6 mg.

E_Y of the control group on day 56 was 603 ± 62 kPa (Fig. 6A). By the completion of the recovery period on day 56, only the 0.004 U/mL group had an E_Y comparable to the undigested control, while E_Y of the 0.015 U/mL group was approximately 50 % of the day 14 values (Fig. 6A). The dynamic moduli of the 0.15 U/mL and 0.004 U/mL groups were 25-30 % greater than the undigested control while the modulus for the 0.015 U/mL treatment was significantly lower than the undigested control ($p < 0.02$; Fig. 6B).

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. 7A). The DNA content of all three chABC groups stayed above the initial day 0 level, which was not observed with multiple high-dose treatments in Study 1 (Fig. 2E – CABC2). The GAG content normalised by DNA for the 0.004 U/mL and the 0.15 U/mL group was
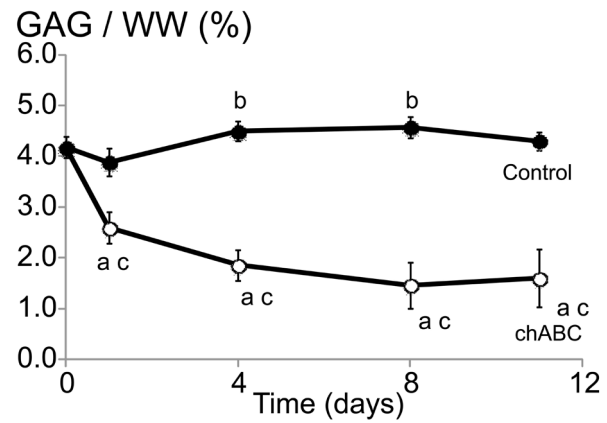

Fig. 4. GAG content normalised by wet weight of mature chondrocyte-seeded constructs (cultured for 42 days) cultured for an additional 11 days in chemically-defined media (control) or media supplemented with 0.15 U/mL chABC on day 0. 'a' denotes $p < 0.05$ between control and chABC group at each time point; 'b' denotes $p < 0.05$ for the control group *versus* day 0 values; 'c' denotes $p < 0.05$ for the chABC group *versus* day 0 values.

more than 2× greater than the control. The GAG content normalised by current day 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; $p = 0.04$; Fig. 7B). The GAG content normalised by wet weight for the 0.15 U/mL group was not significantly different from the control group. To account for the differences in swelling ratio with chABC treatment (Fig. 5B), the GAG content was also normalised by the day 0 wet weight. Only the 0.015 U/mL group had a GAG content normalised by day 0 wet weight that was significantly lower than the control ($p < 0.0001$; Fig. 8A).

By day 56, the 0.004 U/mL group had the highest collagen content (3.25 ± 0.37 %/ww and 21.0 ± 2.3 %/dry
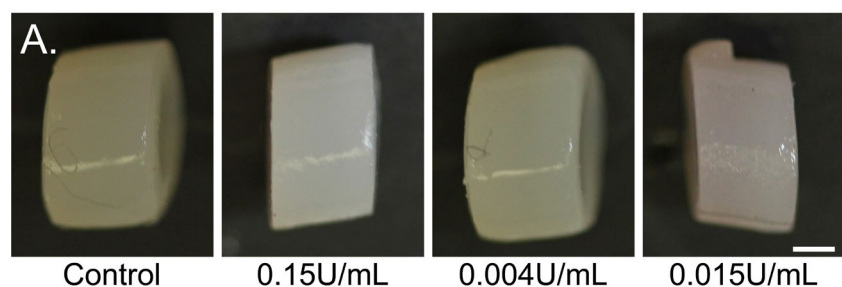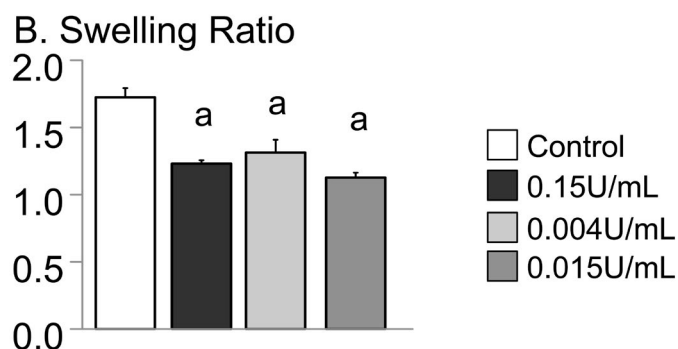

Fig. 5. (**A**) Images of representative samples from day 56 for untreated and chABC-treated constructs. Bar = 1 mm. For Study 2, on day 0, the construct diameter was 4.00 ± 0.09 mm and the wet weight was 26.6 ± 0.6 mg. (**B**) Swelling ratio of constructs at day 56. The initial wet weight of constructs was 26.6 ± 0.6 mg at day 0. 'a' denotes $p < 0.05$ *versus* control.
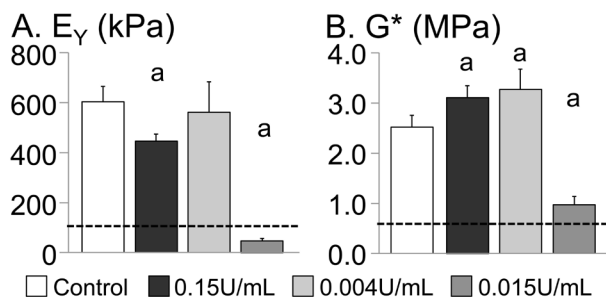
Fig. 6. Compressive mechanical properties of constructs from Study 2 on day 56. (**A**) Equilibrium Young's modulus and (**B**) dynamic modulus measured at 0.5 Hz. Mechanical properties of the very low chABC group (0.004 U/mL for one week) recovered faster than digesting constructs with a higher single dose of chABC (0.15 U/mL applied on day 14). 'a' denotes significant differences from control. The dotted line represents day 14 values (i.e. pre-digestion values).
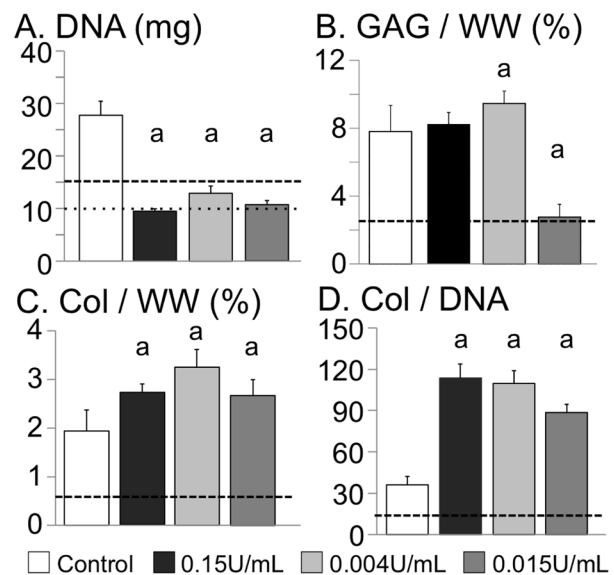
weight), 65 % higher than the control ($p < 0.001$; Fig. 7C). The collagen content of the 0.004 U/mL and the 0.15 U/mL groups was significantly greater than the control for all normalisation methods (i.e. normalisation by DNA, wet weight or dry weight; $p < 0.02$; Figs. 7C,D and 8B). The total construct collagen content of the 0.004 U/mL group was 40 % greater than the control (1.43 ± 0.16 mg for the 0.004 U/mL group and 1.01 ± 0.23 mg for the control; $p < 0.01$).

Discussion

Recapitulating the biochemical composition and structure of native cartilage remains a significant challenge for the field of tissue engineering. Chondroitinase ABC (chABC) has been used successfully to improve collagen fibre structure and composition in engineered cartilage (MacBarb *et al.*, 2013; Natoli *et al.*, 2009b; Responte *et al.*, 2012). Accumulation of GAG occurs much faster than collagen in our engineered constructs, such that the GAG content at day 56 is approximately 3x greater than the collagen content whereas in native tissue there is 2x more collagen than GAG. Previous studies have suggested that excessive GAG deposition early in the culture of engineered cartilage may inhibit collagen deposition due to overcrowding (Bian *et al.*, 2009b). Enhancing the collagen content of replacement tissues is believed to be a necessary condition for developing the high tensile mechanical properties present in native cartilage. Such tensile robustness will be crucial for replacement tissues to withstand the deformation from the high compressive loads within the normal loading of the articular cartilage (Treppo *et al.*, 2000). As the fibrillar collagen network provides nearly all of the tensile support in native cartilage, the aim of this study was to test the hypothesis that more frequent chABC supplementation can improve collagen deposition.
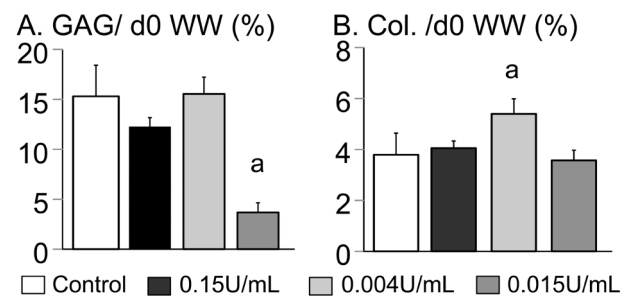

Fig. 7. Day 56 biochemical properties of engineered cartilage from Study 2. (**A**) DNA content, (**B**) GAG content normalised by current day wet weight (i.e. day 56), (**C**) collagen normalised by current day wet weight, and (**D**) collagen content normalised by DNA content. 'a' denotes significant differences from control. The dotted line in (**A**) represents the day 0 DNA level and the dashed line in (**A**), (**B**), (**C**) and (**D**) represents day 14 values (i.e. pre-digestion values).

Fig. 8. Biochemical properties of engineered cartilage from Study 2. (**A**) GAG content and (**B**) collagen content normalised by day 0 wet weight (WW) at day 56. 'a' denotes significant differences from control.

Although prior investigations of a single treatment of chABC caused some cell death, this effect was not significant enough to prevent ECM deposition, allowing for full recovery of mechanical properties and GAG when normalised to the tissue wet weight at the end of the study (Bian *et al.*, 2009b; Natoli *et al.*, 2009a; O'Connell *et al.*, 2012; Responte *et al.*, 2012; Treppo *et al.*, 2000). Based on this prior work, it was hypothesised that by increasing the duration of weekly chABC administrations, collagen deposition would be further enhanced. However, the findings of this study demonstrated detrimental effects of multiple chABC treatments on long-term cell proliferation. Among the various protocols tested in Study 1 and Study 2, only the low dose of chABC supplementation of Study 2 was effective in increasing the collagen content, producing

the least cell death following treatment, and decreasing the culture time needed to recover mechanical properties and GAG content.

The results of Study 1 indicated that multiple high-dose chABC treatments (0.15 U/mL) had an additive detrimental effect on cell viability, causing unrecoverable degradation of the construct ECM, mechanical properties and swelling ratio (Figs. 2 and 3). Interestingly, treatment groups with similar GAG levels (as normalised by current day wet weight) also had similar compressive mechanical properties (notably day 77 of the control and CABC1 groups in Fig. 2). This similarity is in line with previous work indicating that the fixed-charge density of cartilage (due to the high GAG content) contributes strongly to the compressive mechanical properties (Eisenberg and Grodzinsky, 1985; Lai *et al.*, 1991). As a better representation of the loss of ECM synthesis, biochemical composition data were normalised to the initial construct wet weight (i.e. day 0 values; Fig. 3). These results illustrated the severe reduction of both collagen and GAG elaboration when constructs were treated with a single high-dose of chABC. Such results are in line with our earlier work, which demonstrated that constructs supplemented with chABC during the first four weeks of culture had a significantly reduced ECM synthesis capacity (Bian *et al.*, 2009b). This earlier work also observed a reduction in cell viability in groups treated for prolonged periods with high-dose treatments of chABC, potentially contributing to the drastically reduced ECM levels. Taken together, both this earlier work and Study 1 suggest that increasing chABC dosage and frequency can be deleterious for engineered construct growth.

In light of these findings, in Study 2 we attempted to limit the decrease in DNA content following multiple chABC applications by utilising very low doses of chABC. Applying a very low concentration of chABC (i.e. less than 3 % of the high-dose group; Study 2 – 0.004 U/mL group) over a one-week culture period was successful in increasing collagen production without causing a detrimental amount of cell death. At day 56, the DNA content of the very low dose group was greater than the single two-day high dose treatment group (0.15 U/mL group), which suggests that there is a strong correlation between chABC concentration and cell death. Together with Study 1, these results indicate that chABC is not harmless to chondrocytes, and that the concentration, duration and frequency must be balanced to improve collagen deposition, while maintaining cell proliferation, GAG production, and recovery.

The very low chABC treatment group (0.004 U/mL) demonstrated an ability to fully recover compressive mechanical properties, while improving the overall biochemical composition (Figs. 6 and 7). Although not investigated here, previous studies with engineered fibrocartilage tissues have demonstrated an increase in fibre diameter and collagen density following chABC treatment (MacBarb *et al.*, 2013; Responte *et al.*, 2012). Importantly, the collagen content (normalised to day 56 wet weight, DNA, and total mass within the construct) of the very low dose chABC group was 65 % greater than the control group, compared to a 40 % increase in collagen content with the single high-dose chABC treatment (Figs. 7C, D).

These findings suggest that a very low dose of chABC may be more beneficial than a single high-dose treatment for decreasing the total culture time, as well as increasing the overall biochemical composition and tensile mechanical properties of engineered cartilage (Kelly *et al*., 2013).

Previous studies have used multiple high-dose chABC treatments on engineered cartilage to improve collagen production without fully compromising the mechanical integrity of the tissue (Bian *et al*., 2009b; Natoli *et al*., 2009a). In those studies, the second chABC treatment occurred two to four weeks after the first treatment. It is likely that allowing for sufficient extracellular matrix deposition following the first treatment helps to protect the cells during the second enzymatic treatment. The results of Study 2 show that a low-dose protocol eliminates the need for a long recovery time between treatments, which is ideal for developing engineered cartilage as a clinical repair strategy.

Although the collagen content of the very low dose group was 65 % higher than undigested control (3.3 %/ww at day 56), it should be noted that the collagen content of native cartilage is 10-15 %/ww (Klein *et al*., 2007; O'Connell *et al*., 2012; Treppo *et al*., 2000). Therefore, we were able to cultivate engineered cartilage with collagen composition that was approximately 30 % of native values, demonstrating the challenge of recapitulating collagen composition in engineered cartilage *in vitro*. Obtaining native collagen levels may require multiple low-dose treatments over an extended culture duration, particularly for application to engineered human tissues, where the low GAG and collagen synthesis rates typical of adult and often osteoarthritic chondrocytes will further confound obtaining native properties. Furthermore, the collagen alignment of native cartilage tissue is depth dependent, while the collagen alignment of engineered cartilage is random under free-swelling conditions (Bian *et al*., 2009b). Mechanical stimulation has been shown to preferentially align collagen fibres along the positive principle directions of strain (Kelly *et al.*, 2006). It is likely the techniques used here will need to be combined with other culture techniques, such as applied physical stimuli (Kock *et al.*, 2013; Mauck *et al.*, 2000), to develop engineered tissues that replicate the collagen composition and structure of native tissue, and these investigations are the topics of ongoing studies.

Conclusion

Enzymatic digestion of over-deposited GAG with chABC can be an effective method to improve collagen composition in engineered cartilage. This study demonstrated that the concentration and duration of chABC treatments can be optimised to improve collagen deposition and decrease the total culture time needed *in vitro*, while limiting cell death due to enzymatic treatment.

Acknowledgements

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Discussion with Reviewers

Reviewer II: These findings are very intriguing for young bovine cells, which produce an abundance of proteoglycans in culture. Have the authors investigated human cells or adult animal cells to see if the same phenomena and responses are present?

Authors: Current studies are being performed on developing engineered cartilage constructs with human cells (both mesenchymal stem cells and articular chondrocytes). However, as the reviewer notes, GAG production from juvenile bovine cells is much more abundant than human cells. Therefore, the low level of the chABC shown to be beneficial in this study may have to be supplemented later in culture as GAG may not hinder collagen deposition until it is given sufficient time to develop within the constructs.