Introducing Microchannels into Chondrocyte-Seeded Agarose Hydrogels Influences Matrix Accumulation in Response to Dynamic Compression and TGF-β3 Stimulation

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Abstract— **Tissue engineering technologies combining cells with scaffolds are promising strategies for cartilage repair. A recurring problem with scaffold-based therapies is the formation of superior peripheral tissue, resulting in an inhomogeneous tissue construct. Nutrient transfer limitations to the centre of the construct are believed to be responsible for the phenomena. The introductions of channels into a scaffold or hydrogel, or using mechanical loading to improve nutrient transfer, are two potential approaches to overcome this limitation. If both approaches are combined, the mechanical environment within dynamically compressed hydrogels will be modified by the introduction of microchannels into the construct. The objective of this study was to investigate how chondrocytes will respond to this altered mechanical environment.**

Isolated porcine chondrocytes were suspended in 2% agarose. Microchanneled and solid construct cylinders (Ø 6 x 4 mm) were fabricated and maintained in supplemented media containing TGF-β3 (10ηg/ml). Loaded solid and channeled constructs were subjected to a compressive strain amplitude of 10%, for 2 hours/day, 5 days/week, for a duration of 6 weeks.

In the presence of TGF-β3, dynamic compressive loading in solid constructs resulted in an increase in sulphated glycosaminoglycan (sGAG) accumulation. There was a trend towards greater sGAG synthesis in microchanneled constructs; however sGAG accumulation was lower in these groups. While the introduction of microchannels alone may not result in the development of engineered tissue suitable for implantation, it does represent a unique model to investigate chondrocyte mechanobiology.

Keywords— **Dynamic compression, agarose hydrogel, chondrocytes, channeled constructs, TGF-β3.**

I. Introduction

Tissue engineering strategies aim to repair cartilaginous defects through the use of porous scaffold or hydrogel based systems which provide a 3D environment allowing cells to maintain their differentiated phenotype and deposit extracellular matrix (ECM). Agarose hydrogels are commonly used for cartilage tissue engineering applications, as they have been found to support the chondrogenic phenotype and the synthesis of cartilage ECM (1-2). In addition they have been shown to provide a well characterized mechanical environment (3), suitable for investigating cellular responses to biophysical stimuli. The biosynthetic activity of chondrocytes during in vitro cultivation is known to depend on both the biochemical and biophysical stimuli experienced by the cells. Previous studies have shown that the application of dynamic compressive loading to chondrocyte-seeded agarose hydrogels enhances cartilage specific matrix synthesis (4-6).

However a recurring problem with these agarose constructs is the heterogeneous deposition of ECM within them, with typically greater matrix accumulation in the peripheral regions of the construct (7). Kelly et al. (8) reported a greater stiffness in the periphery of both free-swelling and dynamically loaded constructs. Ng et al. (9) also observed a heterogeneous development of material properties in free swelling chondrocyte-seeded agarose disks. It has been suggested that nutrient diffusion limitations are a possible reason for these heterogeneous constructs (10). Dynamic compression can enhance nutrient transport within agarose constructs (11). Altering the architecture of the constructs is another possible way to overcome nutrient transport limitations. Bian et al. (12) obtained a more homogeneous cartilaginous tissue upon the introduction of macroscopic channels throughout the depth of the construct. Buckley et al. (13) found that the introduction of microchannels, in addition to rotational culture, resulted in sGAG accumulation levels in the core similar to those measured in the periphery of solid constructs.

The objective of this study is to investigate the influence of construct architecture and dynamic loading on matrix accumulation within chondrocyte seeded agarose hydrogels. Channels were transversely introduced into cylindrical agarose gels, and constructs were subjected to dynamic loading over a 42 day period. In addition solid, non-channeled constructs were cultured in parallel. Our original hypothesis was that dynamic compressive loading of channeled constructs would enhance nutrient delivery and fluid flow, and therefore lead to a greater and more homogeneous deposition of matrix throughout the construct compared to solid gels or free swelling conditions.

II. Materials and Methods

A. Cell Isolation, Expansion and Hydrogel Encapsulation

Articular cartilage was aseptically harvested from the femoropatellar joints of two 4-month old porcine donors

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(~50kg). Isolated chondrocytes were plated at a seeding density of 8.75×10^6 cells/cm^2 and expanded to passage one (P1).

Chondrocytes were then suspended in DMEM/F12 and mixed with 4% agarose (Type VII, Sigma-Aldrich, Arklow, Ireland) at ~40ºC, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/ml. The agarose/cell suspension was cast in a polytetrafluoroethylene (PTFE) mould, and solid construct cylinders were removed using a 6mm biopsy punch. An equal number of channeled construct cylinders were fabricated via a moulding process as previously described (13). Channels were of 500µm diameter, with a centre-centre spacing of 1mm. Constructs were maintained in 6-well plates in a chemically defined chondrogenic medium (CDM) consisting of DMEM GlutaMAX supplemented with penicillin (100U/ml)-streptomycin (100µg/ml) (all GIBCO, Biosciences, Ireland), 1.5 mg/ml bovine serum albumin (BSA), 100µg/ml sodium pyruvate, 40µg/ml L-proline, 4.7 µg/ml linoleic acid, 50 µg/ml L-ascorbic acid-2-phosphate, 1× insulin-transferrin-selenium, 2.5 µg/ml amphotericin B, and 100nM dexamethasone (all Sigma-Aldrich, Ireland). Media was also supplemented with 10ng/ml TGF-β3 (R&D Systems, Abingdon, UK). After cell encapsulation, constructs were left in free swelling conditions for 72 hours before the addition of TGF-β3, and the initiation of dynamic loading (Day 0). Medium was fully replaced twice a week, with 500µl samples taken from wells for each group (n=2-3) at each medium exchange.

B. Dynamic Compression Application

Intermittent dynamic compression (DC) was carried out in a custom pneumatic based compression bioreactor housed within an incubator as previously described (14). The compression protocol consisted of ~10% strain amplitude superimposed on a 0.01 N/construct preload at a frequency of 1Hz. Constructs were loaded each day for 2 consecutive hours, 5days/week over 42 days. Free swelling (FS) controls were maintained adjacent to the bioreactor during loading periods, in the same amount of medium. Both solid and microchanneled constructs were subjected to both culturing regimes, and constructs were assessed at 0, 21, and 42 days.

C. Biochemical Analysis

The biochemical content of constructs was assessed at each time point. All constructs were cored using a 3mm biopsy punch and separated from the annulus. The wet mass of both the core and annulus was recorded and all samples were subsequently frozen at -85ºC for later analyses. Samples were digested with papain (125µg/ml) in 0.1M sodium acetate, 5mM L-cysteine-HCL, 0.05 M EDTA, pH 6 (all Sigma-Aldrich, Ireland) under constant rotation at 60ºC for 18 hours. DNA content was quantified using the Hoechst Bisbenzimide 33258 dye assay. Proteoglycan content was estimated by quantifying the amount of sGAG in each hydrogel core/annulus using the dimethylmethylene blue dye binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a shark chondroitin sulphate standard. sGAG secreted to culture media was also analysed for each group, with total media volume accounted for. Total collagen content was determined by measuring the hydroxyproline content, using a hydroxyproline to collagen ratio of 1:7.69.

D. Histology and Immunohistochemistry

At each time point, two or more samples per group were formalin fixed, dehydrated, and embedded in paraffin. Sectioning at 5µm produced a cross section perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma-Aldrich, Arklow, Ireland) in 0.1M HCL for sGAG accumulation. Samples were stained with picro-sirius red for collagen deposition. Collagen type I and II deposition were identified by immunohistochemical analysis. A mouse monoclonal collagen type I antibody (1:200; 1.4 mg/ml; Abcam, UK) and mouse monoclonal anti-collagen type II antibody (1:80; 1mg/ml; Abcam) were used as primary antibodies for collagen types I and II respectively. An anti-mouse IgG biotin secondary antibody (1:200; 1mg/ml; Sigma-Aldrich) was used for the detection of both primary antibodies.

E. Statistical Analysis

Statistical analyses were performed using the software package MINITAB 15.1 (Minitab Ltd., Coventry, UK). Groups were analysed for significant differences using a general linear model for analysis of variance with factors of culture time, architecture, dynamic compression, and interactions between these factors examined. Tukey's test for multiple comparisons was used to compare conditions. Significance was accepted at a level of $p<0.05$. Numerical and graphical results are presented as mean ± SD (n=3-4 for each group at each time point).

III. Results

sGAG accumulation was observed to significantly increase in all experimental groups (Fig. 1A). By day 42 dynamically compressed solid groups (DCS) were found to have significantly greater sGAG content (534.7±38.7 µg) compared to all other groups. Dynamically compressed microchanneled groups (DCM) accumulated comparable levels of sGAG as

compared to free swelling microchanneled groups (FSM) (307.18±10.64 μg vs. 304.4±22.2 μg). We also noted that there was no significant difference in sGAG levels between the four groups at the final time point when normalized to DNA (Fig. 1D). DNA content was found to be significantly greater in DCS groups (11.029±1.135 μg), when compared to free swelling solid (FSS) groups (7.566±0.44 μg) after 42 days in culture (Fig. 1C).

We next compared core and annulus accumulation at day 42 (Fig. 3A). All groups were found to have significantly greater sGAG levels in the annulus compared to their corresponding core (μg). This is to be expected as the annulus represents a volume three times that of the core. When normalized to wet weight however (data not shown), each group produced significantly more sGAG in the core than their corresponding annulus. We also found that the FSS, FSM, and DCS groups produced significantly more sGAG in the core than their corresponding annulus at day 42 when normalized to DNA.

At day 42, DCM constructs were found to release the greatest amount of sGAG (1026.6±49.6 μg) to the media ($p<0.05$). We also found at this time point that DCS constructs released significantly more sGAG (921±37.5 μg) to the media than either FSM (812.7±24.4 μg) or FSS (654.08±8.02 μg) constructs ($p<0.05$). Taking into account sGAG both accumulated and released per construct over the 42 day culture period, we calculated that DCM constructs produced the greatest overall sGAG amounts when normalized to DNA (156.3743±15.97704 μg/μg) (Fig. 1F), although the differences were not statistically significant.

Collagen accumulation was observed to increase at each time point (Fig. 1B). DCS groups produced the highest levels of collagen (239.77±21.99 μg) at day 42 ($p<0.05$). Loading was also seen to enhance collagen accumulation in microchanneled groups. When collagen was normalized to DNA, we found no significant difference between groups at day 42 (Fig. 1E).

Construct sections from all experimental groups stained positively with Alcian blue (Fig. 2). DCS constructs exhibited slightly more intense staining for sGAG than FSS constructs, while staining was comparable between the DCM and FSM groups. Less intense staining was observed around the periphery of the constructs. This corresponded to our sGAG biochemical results (%w/w). We also observed that the staining seemed to decrease in intensity further into the core of the construct. A more homogeneous sGAG distribution was observed around DCM constructs. Immunohistochemistry for type II collagen revealed a more homogenous distribution in loaded constructs (Fig. 2).

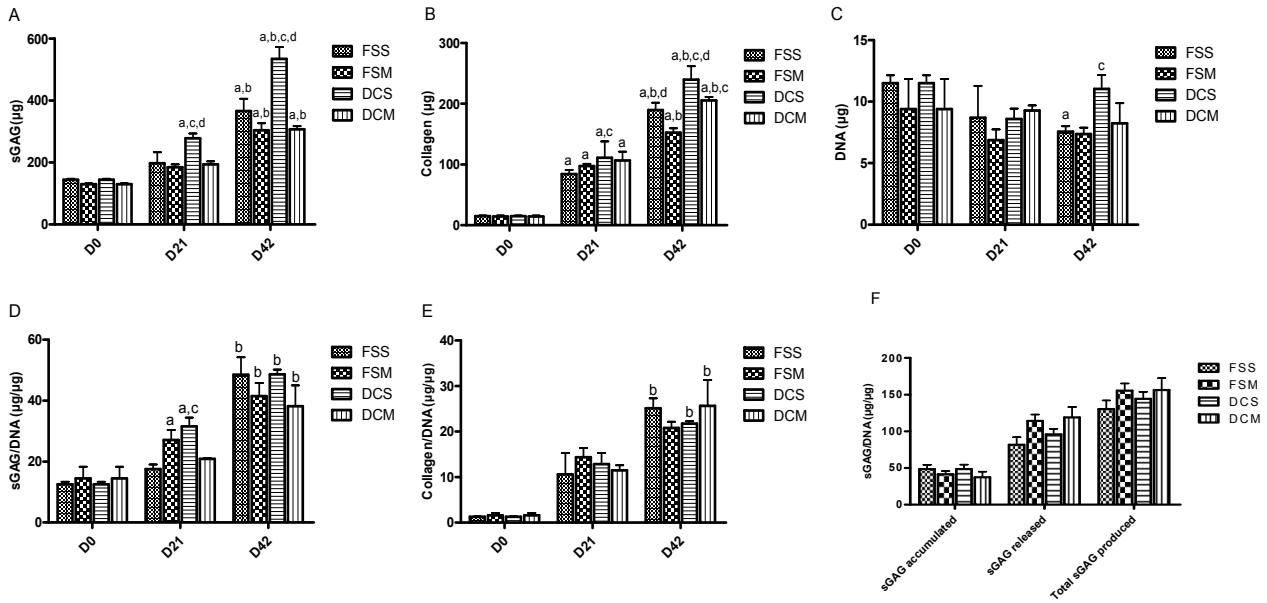

Fig. 1 Biochemical composition of constructs for FSS, FSM, DCS, and DCM groups. (A): sGAG content (μg); (B): Collagen content (μg); (C): DNA content (μg); (D) sGAG/DNA (μg/μg); (E) Collagen/DNA (μg/μg); (F): Total sGAG/DNA accumulated, released, and produced at day 42 (μg/μg). a: $p<0.05$ vs. Day 0; b: $p<0.05$ vs. Day 21; c: $p<0.05$ vs. different culturing conditions with same architecture at same time point; d: $p<0.05$ vs. different architecture with same culturing conditions at same time point.

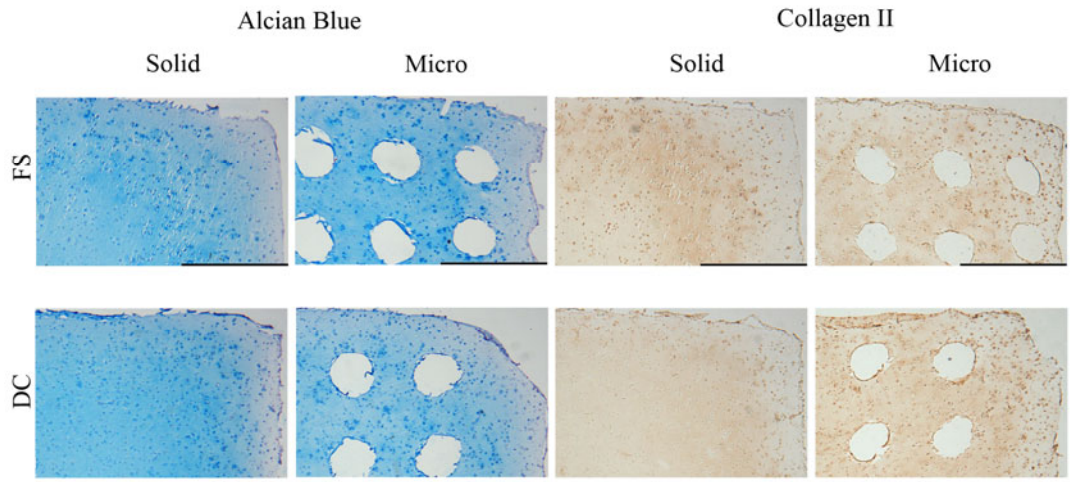

Fig. 2 Alcian Blue staining and type II collagen immunohistochemistry staining of solid and microchanneled constructs subjected to dynamic compression (DC) and free swelling (FS) conditions. Scale bar 1mm. Sections are representative of ¼ of a construct

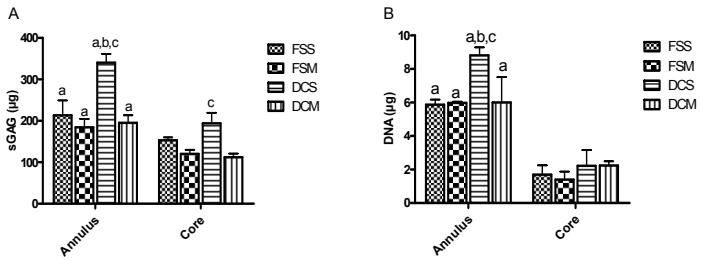

Fig. 3 Biochemical composition of core and annular regions for FSS, FSM, DCS, and DCM groups at D42. (A): sGAG content (µg); (B): DNA content (µg). a: $p<0.05$ vs. core; b: $p<0.05$ vs. different culturing conditions with same architecture in same region; c: $p<0.05$ vs. different architecture with same culturing conditions in same region

IV. Discussion

The purpose of this study was to investigate the influence of dynamic compressive loading and modified construct architecture on the in vitro development of engineered cartilage tissue. It has been previously shown that the biosynthetic activity of chondrocytes depends on the biophysical stimuli experienced by the cells (5). We also observed greater sGAG accumulation in loaded solid constructs compared to all other groups, but we did not find that loading enhanced matrix accumulation in DCM groups. It was seen that loading enhanced cell proliferation in solid gels at day 42, when compared to FSS constructs (Fig. 1C). Comparable sGAG synthesis rates (sGAG/DNA) between all groups at this time point (Fig. 1D) indicate that this superior sGAG accumulation in DCS constructs was due to a higher cell number. The fact that loading maintains cell viability and/or promotes proliferation in solid constructs and not in microchannel constructs may be due to higher levels of fluid flow in loaded channeled constructs. From previous finite element biphasic models for solid and channeled constructs, we predicted high levels of strain and fluid flow at the edges of channels, with pore pressure greatest in the radial spaces separating the channels.

A more homogeneous distribution of sGAG was observed throughout DCM and FSM hydrogels (Fig. 2), suggesting that a modified scaffold architecture can result in a more homogeneous construct. This corresponded to our biochemical results (%w/w), with the DCM and FSM groups presenting the least heterogeneous spatial distribution of sGAG (data not shown). From our histological sections we can see that a significant amount of sGAG is diffusing out into the media, with poor staining around the periphery of the constructs. The fact that both solid and microchannel constructs are releasing sGAG in this manner, for both culturing conditions indicates that agarose may not be an efficient scaffold for retaining ECM. A high oxygen environment at the periphery of the constructs could also be

inhibiting chondrogenesis. We also observed that staining seemed to decrease in intensity the further into the core of the solid construct we passed, once we proceeded past the initial peripheral region, indicating that there may be some nutrient transport limitations towards the core.

Greater sGAG release to the media was observed in loaded constructs, with DCM constructs releasing the greatest amount of sGAG (µg). This can be explained by the microchannels providing a conduit for the diffusion of ECM components into the surrounding media. Taking into account sGAG both accumulated and released per construct over the 42 day culture period (Fig. 1F), we observe a trend towards greater sGAG synthesis in microchanneled constructs, when normalized to DNA. However total sGAG accumulation was lower in this group. This indicates that while the introduction of microchannels may lead to greater ECM synthesis in constructs, the additional ECM components seem to be lost to the surrounding media and not retained by the constructs. Overall the total sGAG accumulation in the construct is reduced by the introduction of channels.

In conclusion this study presents an approach to modifying the local mechanical environment of chondrocytes, in order to understand their basic cellular mechanobiology and engineer cartilaginous tissues. Although a modified architecture may increase the total production of ECM, it does not lead to a greater overall retention in the engineered construct. Further work must look to develop novel approaches to retain sGAG within the developing tissue.

Acknowledgements

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References

1. Benya P, Shaffer J (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 30: 215-224
2. Buschmann M, Gluzband Y, Grodzinsky A et al. (1992) Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. Journal of Orthopaedic Research 10: 745-758
3. Buckley C, Thorpe S, O'Brien F et al. (2009) The effect of concentration, thermal history and cell seeding density on the initial mechanical properties of agarose hydrogels. Journal of the Mechanical Behavior of Biomedical Materials 2: 512-521
4. Mauck R, Soltz M, Wang C et al. (2000) Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. Journal of Biomechanical Engineering 122: 252-260
5. Buschmann D, Gluzband Y, Grodzinsky A et al. (1995) Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. Journal of Cell Science 108: 1497-1508
6. Mauck R, Seyhan S, Ateshian G et al. (2002) Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Annals of Biomedical Engineering 30: 1046-1056
7. Kelly T, Ng K, Ateshian G et al. (2009) Analysis of radial variations in material properties and matrix composition of chondrocyte-seeded agarose hydrogel constructs. Osteoarthritis and Cartilage 17: 73-82
8. Kelly T, Ng K, Wang C et al. (2006) Spatial and temporal development of chondrocyte-seeded agarose constructs in free-swelling and dynamically loaded cultures. Journal of Biomechanics 39: 1489-1497
9. Ng K, Wang C, Mauck R et al. (2005) A layered agarose approach to fabricate depth-dependent inhomogeneity in chondrocyte-seeded constructs. Journal of Orthopaedic Research 23: 134-141
10. Martin I, Wendt D, Heberer M (2004) The role of bioreactors in tissue engineering. Trends in Biotechnology 22: 80-86
11. Albro M, Li R, Banerjee R et al. (in press, 2010) Validation of theoretical framework explaining active solute uptake in dynamically loaded porous media. Journal of Biomechanics
12. Bian L, Angione S, Ng K et al. (2009) Influence of decreasing nutrient path length on the development of engineered cartilage. Osteoarthritis and Cartilage 17: 677-685
13. Buckley C, Thorpe S, Kelly D (2009) Engineering of large cartilaginous tissues through the use of microchanneled hydrogels and rotational culture. Tissue Engineering - Part A 15: 3213-3220
14. Thorpe S, Buckley C, Vinardell T et al. (in press, 2010) The Response of Bone Marrow-Derived Mesenchymal Stem Cells to Dynamic Compression Following TGF-β3 Induced Chondrogenic Differentiation. Annals of Biomedical Engineering: 1-14

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