DYNAMIC DEFORMATIONAL LOADING OF CHONDROCYTE-SEEDED AGAROSE HYDROGELS MODULATES DEPOSITION AND STRUCTURAL ORGANIZATION OF MATRIX CONSTITUENTS

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INTRODUCTION

Articular cartilage tissue engineering has focused on two main goals, providing a maximum of nutrients for tissue growth [1-3] or encouraging tissue development by *in vitro* recapitulation of some of the natural *in vivo* mechanical signals [4-7]. Success of an engineered construct is measured by its ability to replicate the native tissue properties, including its mechanical, histological, and biochemical properties. Early studies using chondrocyte-seeded agarose hydrogels demonstrated that dynamic deformational loading could increase each of these properties, resulting in a stiffer tissue, with a higher number of tissue constituents that are better dispersed within the construct [4,5]. With increases in cell density, growth medium volume, serum concentration, and growth factor addition, however, the correlation between mechanical properties and bulk biochemical constituents (GAG and collagen) becomes less apparent. Recent studies have shown that with similar content of both bulk macromolecules in loaded versus unloaded constructs, mechanical properties can continue to increase with daily dynamic deformational loading [6,8]. These findings have motivated the current study, in which we set out to investigate the effect of long-term loading (12 hours per day) on construct material properties, and to examine matrix organization and regulation of other matrix molecules that may play a role in extracellular matrix structural integrity.

MATERIALS AND METHODS

Cell Culture: Cell-seeded agarose hydrogels were prepared as previously described [4,5]. Briefly, immature bovine chondrocytes were suspended in 2% agarose (Type VII, Sigma) at 60 million cells/ml. Disks (Ø 4.76 x 2.25 mm) were cored and cultured in 100 mm petri dishes (20 to 25 disks per plate) with 30 ml of high glucose DMEM (supplemented with buffers, antibiotics, antimycotics, amino acids, and 50 μg/ml fresh ascorbic acid) at 37°C and 5% CO_2. Media were changed daily. Dynamic loading (DL) was carried out in a custom deformational loading bioreactor [4,5] in a volume of 5 ml DMEM with a loading regime of ~10% strain, at 1Hz, 12 hr on/12 hr off, for 5 days/week. Unloaded, free swelling (FS) controls were maintained in the same amount of media adjacent to the loading device. After loading, disks were cultured in 30 ml of media for overnight culture. Every two weeks, 3-4 disks were removed for analysis, over a 28-day period. **Mechanical Testing**: Mechanical

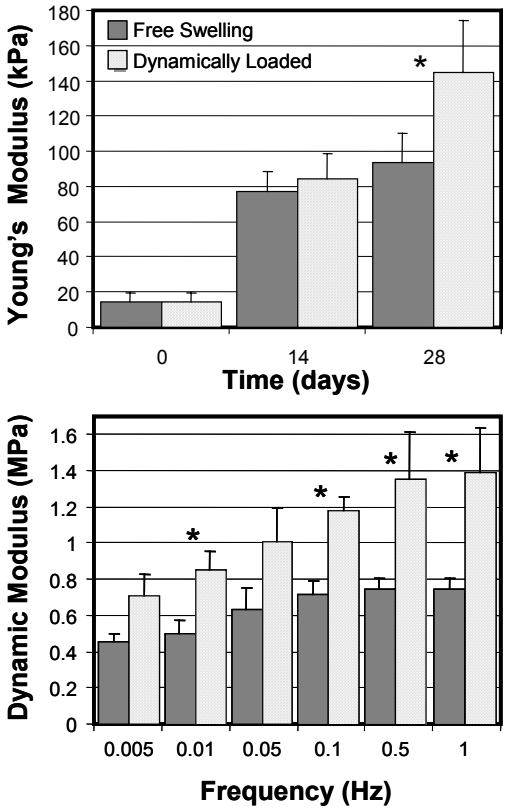

Figure 1 – Young's modulus (top) with time in culture and dynamic modulus (bottom) on day 28. * indicates significant difference from free swelling construct ($p<0.05$, n=3-4).

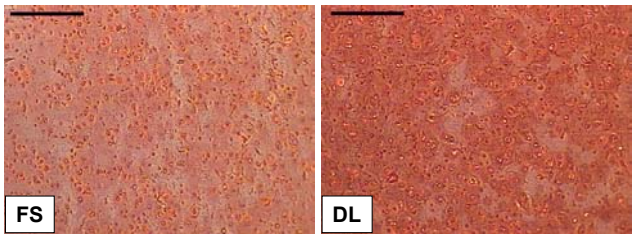

Figure 2 – Safranin O staining of day 28 constructs maintained free swelling (FS) or with dynamic deformational loading (DL). Scale bar: 200 μm.

testing was carried out using stress relaxation tests in unconfined compression with a ramp compression to 10% strain. After equilibrium was reached, a sinusoidal displacement of 40 μm was applied at frequencies ranging from 0.005–1.0 Hz. The Young's modulus and dynamic modulus were calculated from specimen geometry and the resulting load/deformation profiles. Native articular cartilage disks isolated from the same tissue were tested similarly (n=5). **Protein Extraction and Western Blotting**: On day 28, six samples from each condition were weighted wet and frozen at -80°C. Total protein from each disk was extracted with GuHCl extraction as described previously [9]. Protein extracts were precipitated with ethanol and separated by SDS-PAGE, followed by immunoblotting with a polyclonal antibody to COMP F-8. Bands were visualized with chemilluminescence, and their intensity determined and normalized to construct wet weight. **Histology**: Samples were fixed in acid/formalin/ethanol, dehydrated, embedded in paraffin, sectioned, and stained with Safranin O. Scanning electron microscopy (SEM) was carried out at 10 keV on de-paraffinized, gold-coated sections affixed to glass slides. **Statistics**: Statistics were performed using unpaired t-tests assuming unequal variances to compare measured parameters at each time point and each frequency of dynamic testing. Significance was set at α=0.05. All data are reported as the mean ± SD.

RESULTS

By day 28, dynamic deformational loading significantly increased construct material properties. At this point, the Young's modulus reached ~145 kPa for dynamically loaded samples compared to ~94 kPa for free swelling controls (**Figure 1**, $p<0.025$). On day 28, the dynamic modulus of constructs was higher for all dynamically loaded samples compared to free swelling controls (significant for loading frequencies of 1.0, 0.5, 0.1, and 0.01 Hz, $p<0.05$). On day 28, the dynamic modulus (at 1.0 Hz) was ~1.4 MPa for loaded samples compared to 0.75 MPa for free swelling samples (**Figure 1**). These properties compare favorably with the native tissue, which has a Young's modulus of ~277 kPa and a dynamic modulus (at 1 Hz) of ~7.0 MPa. On day 28, GAG content was higher for dynamically loaded samples as exhibited by Safranin O staining (**Figure 2**). Immunoblotting revealed that COMP protein deposition was significantly elevated for dynamically loaded samples compared to free swelling controls (**Figure 3**, $p<0.001$). Preliminary examination of dynamically loaded constructs with SEM revealed lacunar structures in the central region with a more fibrous organization near the radial edge of the construct (**Figure 4**). Local differences in matrix organization may be a function of non-uniform deformation, pressure, fluid flow, and stress fields arising from loading in unconfined compression between impermeable platens.

DISCUSSION

In the agarose culture system, increases in matrix deposition generally lead to increases in construct material properties. In order for matrix constituents to play a role mechanically, however, they must coalesce and form a tissue-spanning structural network. Dynamic deformational loading of chondrocyte-seeded agarose hydrogels has been shown here to increase construct properties compared to free swelling controls. In some cases, however, bulk measures of biochemical constituents (i.e., GAG and collagen) fail to account for the observed increases in mechanical properties [8]. This study demonstrates that other small molecules involved in matrix assembly, such as cartilage oligomeric matrix protein (COMP) [10] are upregulated in response to long-term dynamic loading. COMP is thought to play a role in cell-matrix and matrix-matrix interactions, and may participate in the transduction of physical signals. COMP gene expression has recently been shown to be upregulated in short-term loading of cartilage explants and alginate gels [11]. Further characterization of the distribution of this protein, and other molecules involved in matrix assembly, such as type IX collagen [12], are necessary to determine if these molecules can account for continued increases in construct properties when bulk measures (GAG and collagen) remain unchanged. Examination of structural organization (with SEM) and cross-linking of matrix components may also provide valuable insights into local tissue organization and guide our efforts for the tissue engineering of functional articular cartilage constructs.

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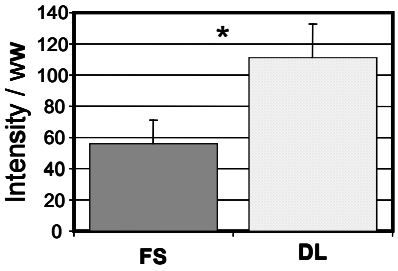
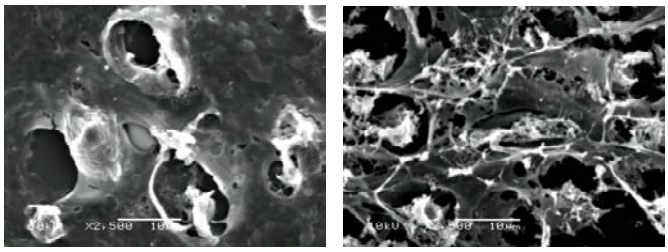


Figure 3 – COMP protein deposition in constructs on day 28 with (DL) and without (FS) deformational loading. * indicates significant difference from free swelling construct ($p<0.05$, n=6).



Center **Edge**

Figure 4 – SEM images of center and radial edge of dynamically loaded construct on day 28. Scale bar: 10 μm.