MECHANOTRANSDUCTION IN MESENCHYMAL STEM CELLS FOR CARTILAGE TISSUE ENGINEERING

NSF Summer Undergraduate Fellowship in Sensor Technologies
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ABSTRACT

Cartilage is a viscoelastic tissue that serves to withstand physiologic loads in the body. The prevalence of cartilage degeneration pathologies such as osteoarthritis in the United States coupled with the tissue's poor native healing capacity call for a cell-based regenerative solution. Current attempts at using multipotent mesenchymal stem cells (MSCs) in a tissue-engineered approach have seen limited success, as MSCs have been shown to deposit extracellular matrix inferior to that produced by adult chondrocytes. In this study, we examine the effects of both short and long-term dynamic deformational loading on MSC extracellular matrix (ECM) production. We also use a novel photocrosslinkable hyaluronic acid (HA) scaffold for MSC seeding. Here, we report that the HA scaffold is capable of supporting MSC growth and chondrogenesis. Moreover, both short and long term dynamic deformational loading applied in conjunction with presence of the chemical morphogen TGF- β3 increase the expression levels of four genes (GalNAc, C4st-1, C4st-2, XT-1) responsible for ECM biosynthesis. Interestingly, prolonged upregulation of the aforementioned genes does not translate to improved mechanical properties or increased proteoglycan content in the ECM. Further studies must therefore be made in order to elucidate the factors responsible for the observed discrepancy and to adjust the current loading regime for optimal ECM biosynthesis.

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1. Background and Introduction:

Articular cartilage is an avascular load-bearing soft tissue that lines the surface of synovial joints. It serves the dual purpose of reducing contact stresses caused by loading and contributing to lubrication of the joint area [1]. These functional characteristics are primarily derived from the biphasic nature of cartilage. Cartilage has previously been modeled as a biphasic tissue composed of an organic solid phase and an interstitial fluid phase. The load-bearing solid phase of cartilage is an organic extracellular matrix (ECM) deposited by chondrocytes that is primarily made up of collagen fibrils and entangled proteoglycan molecules [2]. This matrix is deposited throughout cartilage development and maturation [3]. The response of articular cartilage to mechanical loading is caused by both fluid phase pressurization and solid phase compression [4]. The biphasic nature of cartilage allows it to withstand high levels of physiologic loading. However, pathologies such as osteoarthritis cause deterioration of the ECM, leading to losses in both fluid pressurization and load-bearing capacity. This in turn leads to patient discomfort, joint inflammation, and disability. These symptoms, coupled with the poor intrinsic healing capacity of native cartilage, motivate the development of a cell-based therapeutic strategy for cartilage repair.

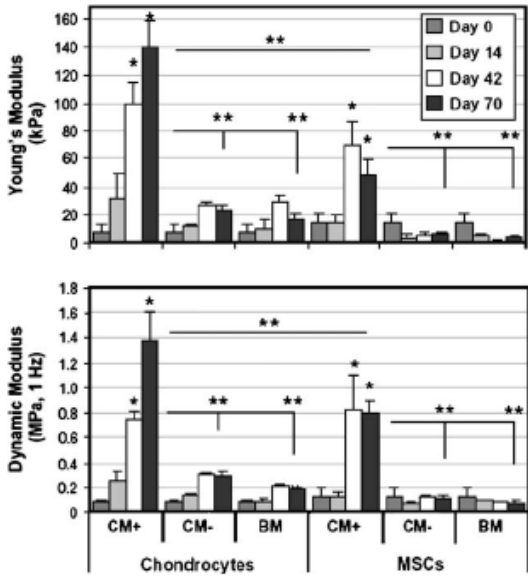


Fig. 1. ECM produced by MSC-laden constructs shows inferior dynamic and equilibrium moduli to that produced by chondrocyte-laden constructs [9]

Recently, tissue engineering (TE) has emerged as the dominant paradigm for cell-based strategies. TE relies on the interaction between cells, scaffolds, and the extracellular environment to create a biological construct that functionally mimics the tissue of interest. Early studies of autologous chondrocyte transplantation have proven largely inconclusive. This lack of success is partially due to the limited availability of chondrocytes and their tendency to dedifferentiate with monolayer expansion [5]. Furthermore, autologous chondrocyte harvest may further weaken the defect site, accelerating the onset of various cartilage pathologies. For these reasons, mesenchymal stem cells (MSCs) have been viewed as an increasingly attractive cell source for cartilage tissue engineering. MSCs are a multipotent population of self-renewing cells that are easily obtained from bone marrow aspirates [6]. They have shown the capability to differentiate into fat, bone, tendon, cartilage, and muscle cells depending on their developmental environment [7]. They have also been shown to undergo chondrogenic induction when in the presence of chemical morphogens from the transforming growth factor β superfamily (TGF- β) [8]. MSCs that acquire a chondrogenic phenotype are characterized by increased gene expression and protein deposition of both type II collagen and aggrecan molecules. MSCs also lose much of their morphology and take on a rounded appearance upon chondrogenic differentiation [9].

Despite the development of a chondrogenic phenotype upon application of TGF- β, Mauck et. al. have shown that matrix deposited by these MSCs is less robust than that of adult chondrocytes.

Specifically, both the mechanical properties and biochemical content of the MSC-generated matrix are inferior to that produced by chondrocytes [9]. These results suggest differences in chondrogenesis-related gene expression between chondrocytes and MSCs despite the presence of a chondrogenic signal.

Thus far, our laboratory has identified four genes responsible for matrix biosynthesis have been determined to be differentially expressed in primary chondrocytes and MSCs. These genes, xylosyltransferase-1 (XT-1), GalNAc4,6S-disulfotransferase (GalNAc), chondroitin-4-sulfotransferase-1 (C4st-1), and chondroitin-4-sulfotransferase-2 (C4st-2), are all responsible for proteoglycan synthesis and organization and play a crucial role in ECM deposition. The biochemical and mechanical effects of modulating the expression levels of these genes are as yet undetermined.

A variety of methods have been used in order to create a tissue engineered construct that mimics biological tissue. While early studies on MSC chondrogenesis focused on the use of high density cell pellet cultures, the TE paradigm relies on the use of scaffolds for structural support and cell signaling [8]. An ideal scaffold must also degrade with the formation of natural ECM. A multitude of biomaterials have been explored for scaffold applications, including alginate, fibrin, collagen, peptide hydrogels, and polyester films [11, 12]. While these materials all demonstrate the ability to encapsulate chondrocytes or MSCs to some degree, there is a growing need to develop scaffolds that promote optimized neocartilage formation. Agarose hydrogels have thus far emerged as the staple material in cartilage tissue engineering. The viscoelastic nature of a hydrogel material causes it to behaviorally mimic natural cartilage in response to loading. In addition, both chondrocytes and MSCs have shown a many-fold increase in equilibrium modulus and dynamic stiffness when cultured in agarose hydrogels [13]. However, the lack of both purifiability and degradability of the naturally-derived agarose prevent the material from being a realistic option to be used for implantable cartilage constructs. Recently, cell photoencapsulation has been explored as a potential approach towards synthesizing an implantable regenerative construct [14]. Photopolymerizable hydrogels possess a unique advantage over other biomaterials with their ability to fill irregularly shaped defects. Photopolymerization occurs when a polymer-photoinitiator solution is exposed to low intensity ultraviolet light [15]. This process was first used to synthesize a poly(ethylene) glycol (PEG) hydrogel and has since been applied to numerous other biomaterials.

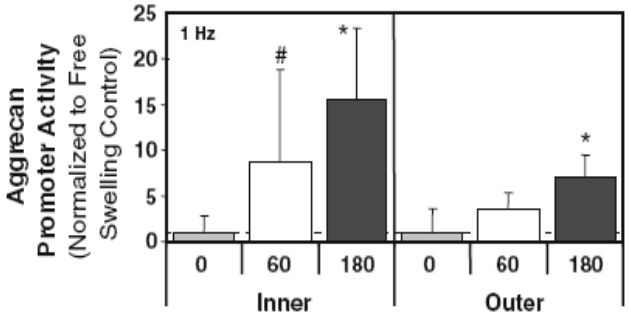


Fig. 2. Aggrecan gene expression levels are increased over time in response to 1 Hz dynamic loading in both inner core and outer annulus regions [9]

Another possible material being considered for bioencapsulation is hyaluronic acid (HA), a natural biomaterial comprised of alternating residues of N-acetyl-D-glucosamine and D-glucoronic acid. A natural component of ECM, HA is believed to be crucial in tissue remodeling and has demonstrated high-affinity binding to cell surface receptors CD44, ICAM-1, and RHAMM [16]. HA is an attractive scaffold material both for its readily modifiable structure and

for its cell-mediated degradative properties [17]. Photopolymerized methacrylated HA scaffolds have been shown to encapsulate articular and auricular chondrocytes in both *in vitro* and *in vivo* settings [15,18]. HA constructs with encapsulated chondrocytes have exhibited mechanical and biochemical properties comparable to that of native cartilage [19]. Gerecht et. al. have also demonstrated that HA hydrogels support the maintenance of differentiation potential of encapsulated human embryonic stem cells [20]. However, the ability of HA to support the growth and chondrogenesis of MSCs after photoencapsulation is as yet undetermined.

In addition to scaffold characteristics, the extracellular environment also plays a crucial role in ECM synthesis and turnover. Specifically, mechanical signaling contributes significantly to cartilage development. In a physiological context, joint regions subjected to greater levels of loading have shown heightened concentrations of matrix proteoglycans [21]. Moreover, mechanical loading of cartilage constructs has been shown to affect gene expression and matrix deposition in both MSCs and adult chondrocytes [22, 23, 24]. It is believed that increased fluid flow activates a signal cascade, leading to gene expression modulation and subsequent biochemical and mechanical changes in ECM. However, the duration and type of mechanical loading is crucial in the degree of ECM modulation. While static ramp-and-hold loading protocols have triggered catabolic responses in chondrocyte ECM, both dynamic loading and dynamic shear of cartilage constructs has resulted in upregulation of collagen type II and aggrecan gene expression levels in addition to increased matrix biosynthesis [21]. Szafranski et. al. have shown that genes responsible for proteoglycan synthesis are upregulated after dynamic compression [25]. Furthermore, Huang et. al. have suggested that dynamic mechanical compression induces changes in MSC gene expression even in the absence of the morphogen TGF- β3 [26].

In this present series of studies, we sought to address the ability of a 2% hyaluronic acid hydrogel to support MSC growth and chondrogenesis. We also addressed the question of whether short term dynamic loading modulated expression levels of matrix biosynthesis genes XT-1, C4st-1, C4st-2, and GalNac. Furthermore, we addressed biochemical changes in ECM after long-term dynamic loading. Here, we report that HA represents a viable scaffold that supports MSC growth and chondrogenesis. In addition, we report that short-term dynamic loading of MSC-laden constructs upregulates XT-1, C4st-1, C4st-2, and GalNac in both HA and agarose hydrogels. We also report that long-term dynamic loading has a deleterious effect on both the mechanical properties and biochemical content of MSC constructs. This phenomenon was observed in both HA and agarose seeded constructs. Further studies are required in order to determine the factors behind the observed inconsistency behind short-term gene expression and long-term biochemical changes.

2. Materials and Methods

2.1 Experimental Design

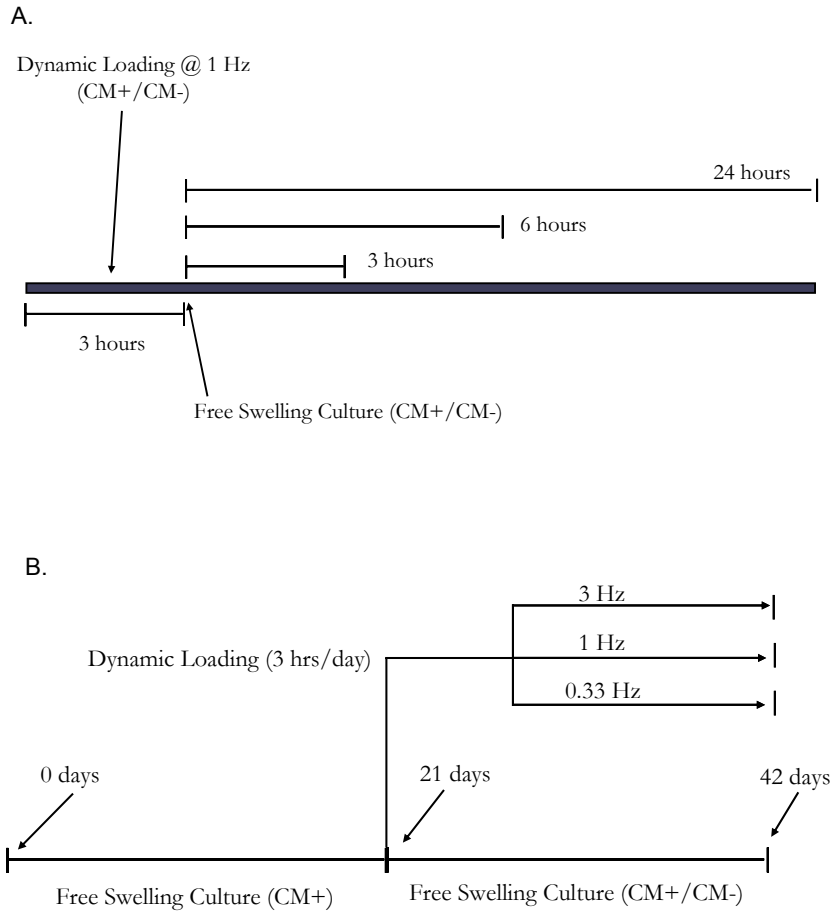


Fig 3. Schematic of TGF- β3 and loading durations for A) Study 2 and B) Study 3. Hash marks indicate harvest points.

Three separate studies are reported here. Study 1 reported the ability of 2% HA to support MSC growth and chondrogenesis when compared to a traditional 2% agarose hydrogel; Study 2 reported short-term gene expression changes in MSC-laden constructs in response to dynamic deformational loading; Study 3 reported long-term gene expression and biochemical content changes in MSC-laden constructs in response to prolonged and repeated cycles of dynamic deformational loading. In each study, individual cell isolations were pooled from multiple donors.

In each of the three studies mentioned above, four variables were manipulated to achieve specific conditions for cell growth and ECM deposition. These variables were (1) frequency of dynamic deformational loading, (2) time course for application of TGF- β3, (3) amount of time left in culture after dynamic deformational loading, and (4) type of hydrogel used. The schematic of these studies is outlined in Figure 4. Specifically, TGF- β3 was administered from day 0 in Study 1 in both agarose and HA constructs. The constructs were left for 21 days in culture before harvest. In Study 2, HA constructs were dynamically loaded for three hours one day after MSC encapsulation either in the presence or absence of TGF- β3. Constructs were subsequently cultured in the same media under free swelling conditions and harvested at 0, 3, 6, and 24 hours after the end of loading. In Study 3, MSCs were seeded in both agarose and HA hydrogels and

allowed to culture for 21 days in presence of TGF- β3 in order to acquire a chondrogenic phenotype. Constructs were then subjected to dynamic deformational loading for an additional period of 21 days either in the presence or absence of TGF- β3 before harvest.

2.2 MSC Isolation and Expansion

Bone marrow derived MSCs were obtained from freshly slaughtered 3-6 month old calves (Fresh Farms Beef, Rutland, VT, USA). Marrow samples were pooled and placed in high glucose Dulbecco's Modified Eagle Medium (hgDMEM) supplemented with 1 x penicillin/streptomycin/fungizone (PSF) and 300 U/mL heparin. Marrow samples were then vortexed and centrifuged at 300x*g* for 5 minutes. The resulting pellets were then plated on 30 cm tissue culture plates (Falcon Becton Dickinson Labware) and cultured in fresh changes of serum containing media (hgDMEM, 10% fetal bovine serum [FBS], 1 x PSF) twice a week in 37° C with 5% CO_2. Upon reaching confluence, MSCs were subcultured at a 1:3 expansion ratio in 60 cm tissue culture plates, with cultures up to passage three being used for all studies.

2.3 MSC Encapsulation

MSCs (P2, P3) were plated in 60 cm plates (Falcon Becton Dickinson Labware) and allowed to reach confluency. MSCs were washed twice with 1 x phosphate buffered saline (PBS) and treated with 10 mL of trypsin-EDTA for five minutes at 37° C and 5% CO_2. Once cell detachment was observed, the reaction was neutralized in 20 mL SCM media. The resulting cell suspension from all plates was pooled and centrifuged at 300x g for five minutes, resuspended in SCM media, and counted using hemocytometer.

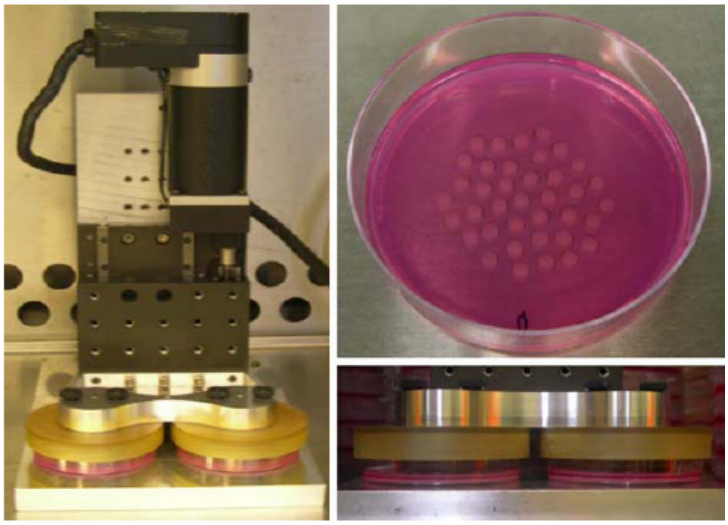


Fig. 4. A compressive loading bioreactor was used to apply a 2% tare followed by 10% peak-to-peak deformation at either 0.33, 1, or 3 Hz. Constructs were placed in Petri dishes and submerged in CDM+ or CDM- media throughout both dynamic compression and free-swelling culture.

Part of the MSC suspension was centrifuged again and resuspended in chemically defined media (CDM-; 1 x PSF, 0.1 mM dexamethasone, 50 mg/mL ascorbate 2-phosphate, 40 mg/mL L-proline, 100 mg/mL sodium pyruvate, 1 x ITS pre-mix) at 40 million cells / mL. This new suspension was mixed with an equal volume of 4% type VII agarose and cast in a 2.25 mm gel slab. 4 mm diameter disks were punched from this gel slab using a biopsy punch and allowed to culture in CDM- media overnight before beginning subsequent studies.

MSC encapsulation in an HA hydrogel was achieved by UV-sterilizing approximately 100 mg HA (Burdick Lab). The sterilized HA was dissolved in I-2959 photoinitiator solution at 37° C to obtain a final 2% HA 0.05% I-2959 solution. The remaining cell suspension was centrifuged and

resuspended in the HA solution to obtain a final cell concentration of 20 million cells per mL. The new MSC suspension was cast in a 2.25 mm thickness mold and exposed to high-wavelength UV light for ten minutes. 4 mm diameter disks were punched from the resulting gel slab using a biopsy punch and allowed to culture in CDM- media overnight before beginning subsequent studies.

2.4 MTT Assay

MSC metabolic activity was quantified via staining with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent). Constructs were first weighed, then placed in MTT reagent diluted 1:10 with DMEM and allowed to react for 60 minutes in 37° C and 5% CO_2. Constructs were then washed twice with 1 x PBS, resuspended in dimethyl sulfoxide (DMSO), and frozen in -20° C. 150 µL of MTT cell suspension for each sample was aliquoted into a microplate well. The microplate was subsequently read and the resulting values normalized to construct wet weight.

2.5 Dynamic Loading

Dynamic deformational loading was achieved using a custom compression bioreactor to apply defined cyclic compressive loads to constructs in a Petri dish [22]. Construct placeholders were made by uniformly covering each sterilized dish bottom with 4% agarose. A 5 mm biopsy punch was used to punch holes in the agarose layer. Both agarose and HA constructs were subjected first to a 2% tare and then a 10% peak-to-peak deformational load. Constructs were loaded on three alternating days of each week for 180 minutes at 0.33 Hz, 1 Hz, or 3 Hz. After day 7 of dynamic loading, 12 HA constructs were removed from culture and measured using digital micrometer. Both tare and deformational load for remaining constructs were adjusted to reflect new thicknesses of the swollen HA constructs.

2.6 Real Time PCR

Gene expression levels were quantified via real time polymerase chain reaction (RT-PCR; Applied Biosystems 7300 Real-time PCR machine). Three replicate constructs per condition were first pooled and frozen in TRIZOL reagent at -80° C. RNA extraction was performed by thawing and grinding constructs. This was followed by phase separation using double chloroform extraction. The purified RNA sample was then sequentially centrifuged at 12,000 rpm at 4° C for 10 minutes and resuspended in isopropanol and 70% ethanol before finally being centrifuged and resuspended in 30 mL DEPC water at 65° C for 5 minutes. RNA quantification was achieved using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). RNA yields ranged from 92-525 ng/µL, with 280/260 ratios > 1.7 for all samples.

Complementary DNA (cDNA) synthesis was performed for up to 2000 ng of RNA product with First Strand cDNA Synthesis Kit (Invitrogen) using random hexamers as per manufacturer's instructions. The final cDNA product was diluted in DEPC water to achieve a final volume of 100 µL. RT-PCR of cDNA samples was carried out using bovine chondrocyte cDNA at concentrations of 50, 5, 0.5, 0.05, and 0.005 ng/µL as standards. Individual cDNA samples were mixed with DEPC water, SYBR Green PCR Master Mix (Qiagen), and 300 nM of forward and reverse primers for either GAPDH, type II collagen, GalNac, C4st-1, C4st-2, or XT-1 to make up

the PCR reaction mixture. Gene transcript levels were normalized to corresponding GAPDH levels to account for varying amounts of cDNA synthesized.

2.7 Mechanical Testing

Mechanical testing of long-term agarose and HA constructs from Study 3 was carried out using custom apparatus [9]. Constructs were subjected to unconfined compression between two incompressible surfaces in 1 x PBS at room temperature. Construct widths and thicknesses were measured using digital micrometer. Constructs were first tested in creep under a tare load of 0.02 N and allowed to equilibrate for approximately 300 seconds. Constructs were then subjected to a stress relaxation test with 10% static deformation applied over 200 seconds and allowed to equilibrate for an additional 1000 seconds. The equilibrium modulus $E_{Y\sim}$ was determined via the following:

$$E_{Y\sim} = \frac{\frac{F_{eq} - F_I}{A}}{\varepsilon}$$

in which F_{eq}, F_I, ε, and A represent the equilibrium force, the initial force, the strain level, and the cross-sectional area of the construct respectively. After testing, constructs were frozen at -20° C for biochemical analysis.

2.8 Biochemical Analysis

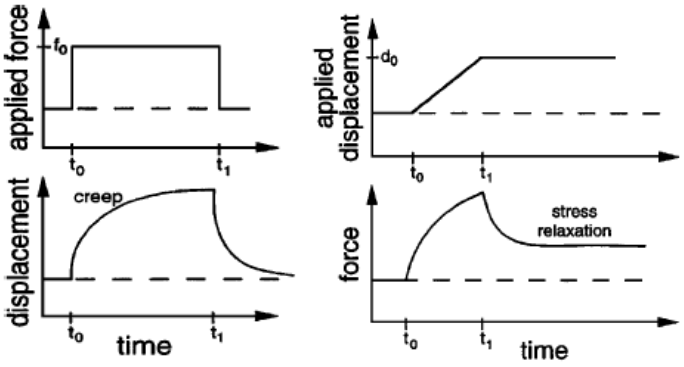


Fig 5. Mechanical testing was achieved by applying a constant load creep test, followed by continuous displacement stress relaxation test [1].

After mechanical testing, the total DNA, collagen, and proteoglycan content present in constructs for specific conditions was determined using biochemical assays. The wet weight of each construct was first measured. Constructs were then digested in papain for 16 hours at 60° C. *Ortho*-hydroxyproline (OHP) content was quantified through acid hydrolysis followed by reactions with Chloramine T and dimethylaminobenzaldehyde. Calculated OHP content was then translated to total collagen content using a 10:1 collagen:OHP conversion ratio. Total proteoglycan content was approximated to sulfated glycosaminoglycan (sGAG) content and measured using the 1,9-dimethylmethylene blue dye binding assay. Total DNA content was quantified using PicoGreen dsDNA Quantification Kit (Molecular Probes) as per manufacturer's instructions. Total collagen, sGAG, and DNA content was normalized to construct wet weight.

2.9 Histology

Constructs for histology were fixed in paraformaldehyde at 4° C for at least 24 hours after harvest. Samples were then dehydrated with successively graded ethanol and Citrisolv treatments, and embedded in paraffin. Paraffin-embedded samples were cut into 6-µm thick

sections and stained with Alcian Blue to visualize proteoglycan content. Stained sections were visualized using inverted microscope.

3. Results

3.1 Characterization of MSC Growth and Chondrogenesis in HA Hydrogels

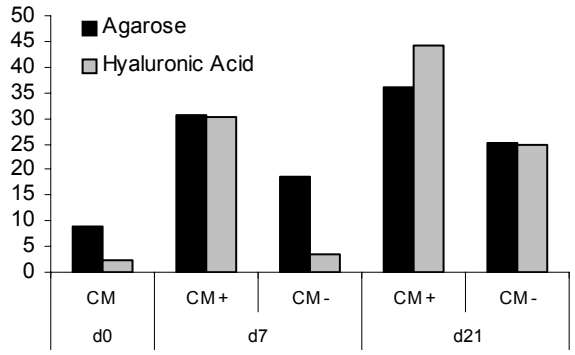


Fig. 6. MTT results indicate no significant different in cell growth between MSC-laden HA and agarose hydrogels over a period of 21 days.

MSCs encapsulated in HA hydrogels and cultured in free swelling conditions exhibited rates of growth and matrix deposition comparable to that seen in free swelling agarose hydrogels (Fig 6). MTT assay results predictably showed that cell numbers rose steadily from day 0 to day 21 in both HA and agarose hydrogels regardless of TGF- β3 application. Direct comparison between HA and agarose hydrogels also showed no noticeable difference in cell growth at all time points, especially in the presence of TGF- β3 (CM+).

Chondrogenesis, quantified as a measure construct collagen and proteoglycan content, was also comparable between agarose and HA hydrogels. Both collagen and proteoglycan levels rose in long term free swelling culture in the presence of TGF- β3. Although mild biochemical content variation existed with respect to individual constructs, mean wet weight percentages for total collagen content were 0.63 ± 0.36% for agarose and 0.69 ± 0.14% for HA constructs (Fig 7B). GAG content varied more dramatically between individual constructs, with mean percentages of 1.30 ± 0.19% for HA and 0.86 ± 0.67% for agarose (Fig 7A).

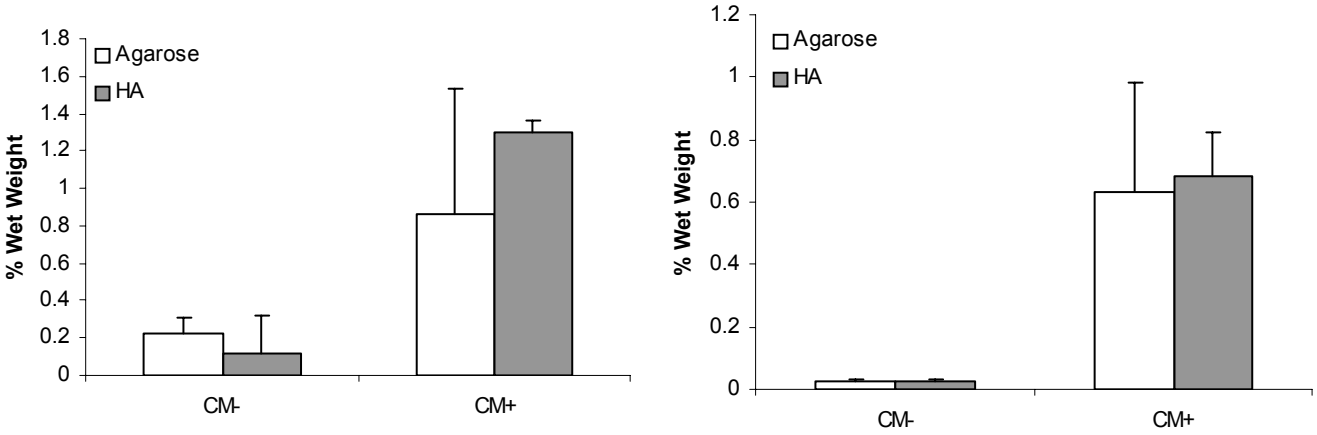


Fig. 7. A) Proteoglycan and B) total collagen content of MSC-laden constructs are comparable between agarose and HA hydrogels (n = 3). All transcript expression levels are normalized to GAPDH.

3.2 Temporal Gene Expression of Matrix Biosynthesis Genes in Response to Short Term Loading

MSC-laden HA constructs were first subjected to 180 minutes of dynamic compressive loading at 1 Hz in CM+ or CM- media, and then allowed to culture in free swelling conditions until being harvested. Constructs were removed at 0, 3, 6, and 24 hours after dynamic loading. Col-2, GalNAc, and C4st-1 gene transcripts were initially quantified at all time points. Col-2 transcripts showed a dramatic increase between 6 and 24 hours in CM+ media after both free swelling culture and dynamic loading. In addition, exposure to dynamic loading increased Col-2 expression by approximately 2.5-fold. C4st-1 transcripts exhibited similar behavior in response to both dynamic loading and TGF- β3 exposure. While C4st-1 upregulation due to TGF- β3 was detectable even at three hours, the highest levels of gene transcription were observed 24 hours after dynamic loading in CM+. Dynamically loaded CM+ C4st-1 transcript levels after 24 hours were increased 3-fold over corresponding free swelling transcript levels (Fig 8B). Conversely, GalNAc gene transcripts were dramatically downregulated in response to TGF- β3. Dynamic loading increased expression levels of GalNAc most markedly in CM- media conditions, although GalNAc transcript upregulation was observed to some degree at virtually all time points regardless of media conditions (Fig 8C, 8D).

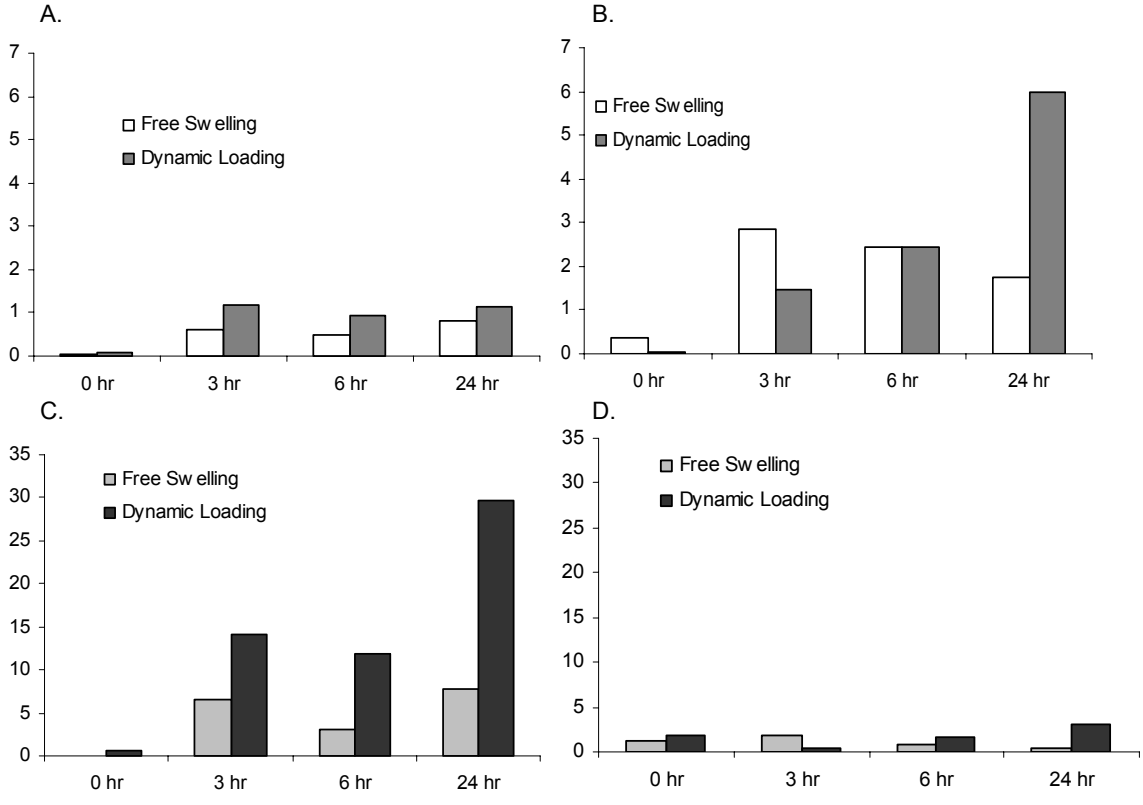


Fig 8. Gene expression levels of C4st-1 in A) CM- and B) CM+ media show that dynamic loading upregulates transcripts at all time points in both media conditions. A similar trend can be observed for GalNAc transcripts in C) CM- and D) CM+ conditions

C4st-2 and XT-1 expression levels were also quantified for 0 and 24 hours in both media conditions. Over 24 hours, C4st-2 transcripts were increased three-fold in CM- media and over

two-fold in CM+ media after application of dynamic loading (Fig 9A). Similarly, XT-1 transcripts were increased approximately two-fold in both CM- and CM+ conditions in response to dynamic loading (Fig 9B). Both genes also showed increased transcription in CM+ conditions over CM- conditions (Fig 9A, 9B).

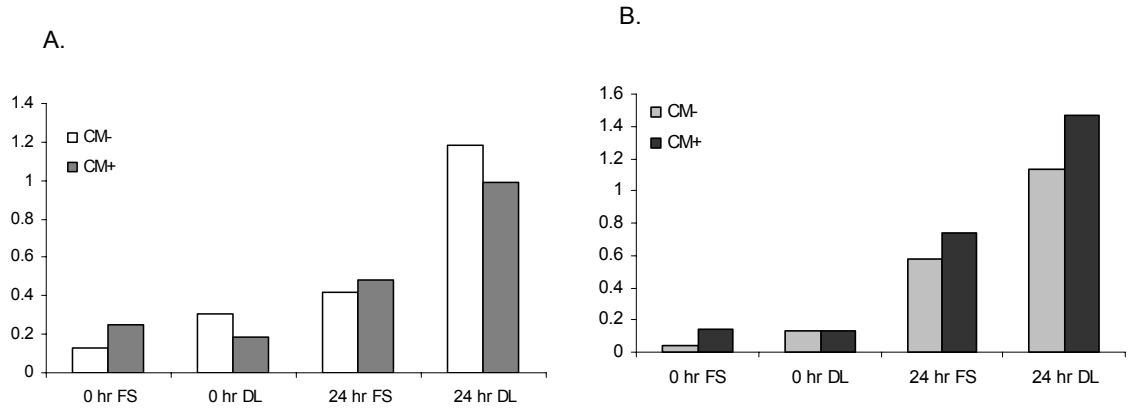


Fig 9. Gene expression levels of A) C4st-2 and B) XT-1 also show upregulation of transcripts levels 24 hours after dynamic loading at 1 Hz. All transcript expression levels are normalized to GAPDH.

3.3 Temporal Gene Expression of Matrix Biosynthesis Genes in Response to Long Term Loading

MSC-laden HA and agarose constructs were harvested at 0 and 21 days after free swelling culture in addition to 21 days after dynamic loading at 1 Hz. Col-2, GalNAc, C4st-1, C4st-2, and XT-1 gene transcripts were quantified using RT-PCR and normalized to GAPDH levels. HA constructs showed significant upregulation in all quantified transcripts in response to prolonged dynamic loading regardless of media conditions. Col-2 levels were increased from 12.98 to 39.24 in response to dynamic loading in CM+ media. Constructs cultured an additional 21 days after removal of TGF- β3 expressed Col-2 levels of 1.98 and 2.06 for free swelling and dynamically loaded constructs respectively (data not shown). These gene expression levels were down from 7.31 as found after 21 days of CM+ free swelling culture. In C4st-1, dynamic loading increased HA constructs from 1.19 to 2.68 in CM+ and from 0.30 to 0.79 in CM- conditions, as compared to 0.34 after 21 days of free swelling culture (Fig 10C). C4st-2 transcripts increased from 0.50 to 0.59 in CM- and 0.46 to 0.87 in CM+ conditions, as compared to 0.55 after 21 days in CM+ free swelling conditions (Fig 10D). GalNAc expression increased with long-term dynamic loading from 0.32 to 0.93 in CM- conditions and 0.07 to 0.41 in CM+ conditions in comparison to 0.05 after 21 days of free swelling culture (Fig 10A). Finally, XT-1 gene expression increased from 0.30 to 0.62 in CM- and from 0.34 to 1.36 in CM+ conditions, in comparison to 0.41 after 21 days of CM+ free swelling conditions between 6 and 24 hours (Fig 10B).

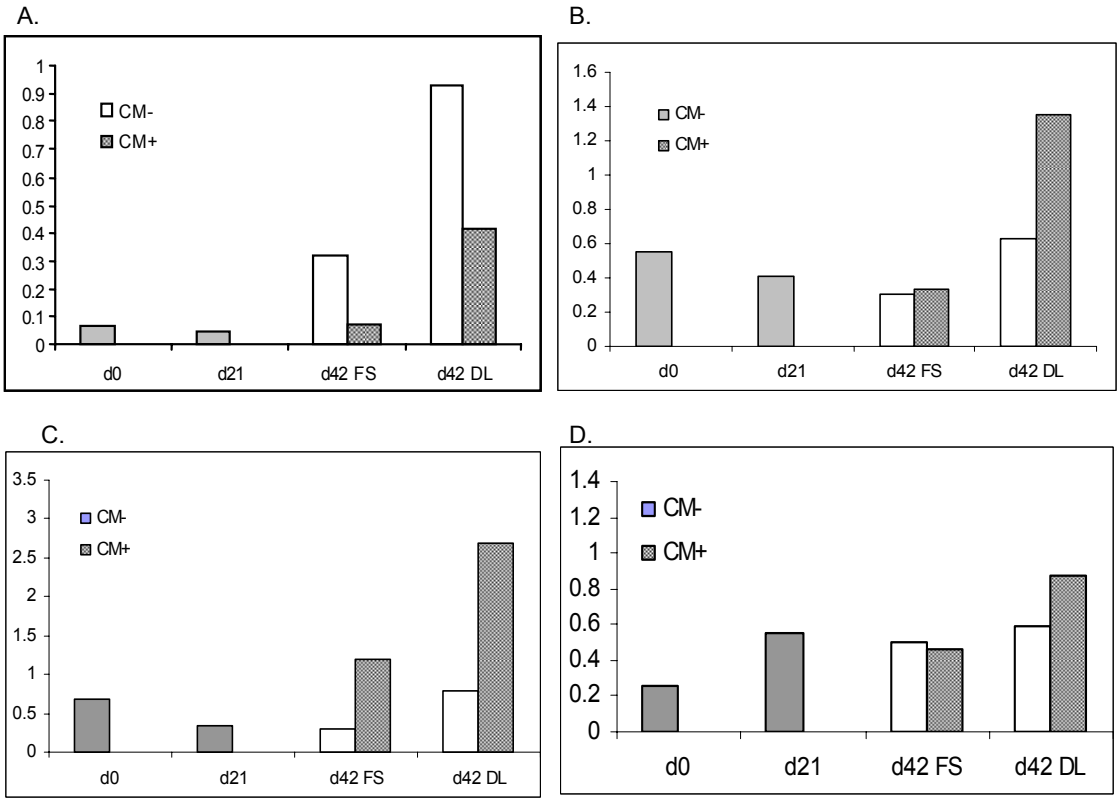


Fig 10. Long term gene expression levels of A) GalNAc, B) XT-1, C) C4st-1, and D) C4st-2 in agarose hydrogels show moderate levels of gene upregulation in response to extended dynamic loading. All transcript expression levels are normalized to GAPDH.

Agarose hydrogels displayed similar trends in gene transcript modulation. Col-2 expression levels remained unchanged in CM- media at approximately 4.5 and increased from 5.57 to 13.22 in response to dynamic loading in CM+ media. This is in comparison to a Col-2 level of 5.0 at day 21. C4st-2 was upregulated from 0.22 to 0.57 in CM- and from 0.14 to 0.46 in CM+ conditions while C4st-1 was upregulated from 0.17 to 1.13 in CM+ conditions and downregulated from 0.85 to 0.58 in CM- conditions. Day 21 free swelling CM+ gene expression levels for agarose were 0.45 and 0.37 for C4st-2 and C4st-1 respectively (Fig 11C, 11D). GalNac expression levels changed from 0.04 to 0.19 in CM- and from 0.011 to 0.096 in CM+ conditions in response to dynamic loading, while XT-1 levels changed from 0.22 to 0.31 in CM- and 0.10 to 0.22 in CM+ conditions. Day 21 CM+ free swelling values for GalNAc and XT-1 were 0.049 and 0.404, respectively (Fig 11A, 11B).

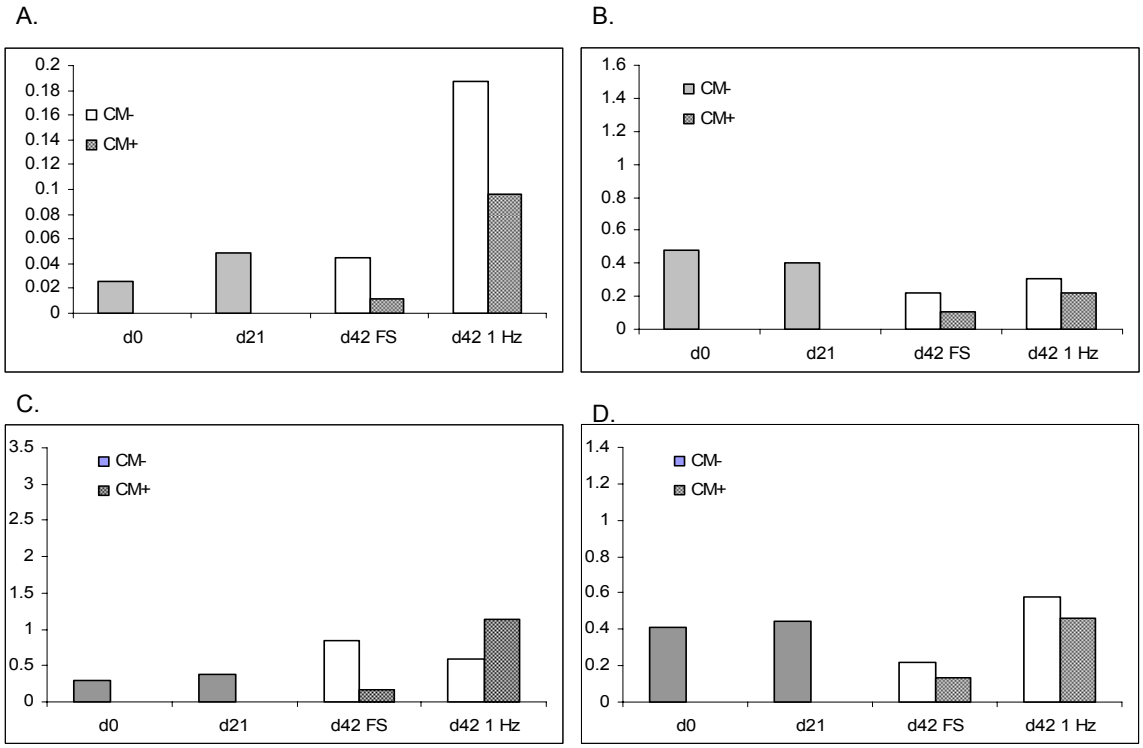


Fig 11. Long term gene expression levels of A) GalNAc, B) XT-1, C) C4st-1, and D) C4st-2 in agarose hydrogels show moderate levels of gene upregulation in response to extended dynamic loading. All transcript expression levels are normalized to GAPDH.

3.4 Mechanical Properties of HA and Agarose Constructs in Response to TGF- β3 Removal and Long Term Loading

HA constructs were subjected to the treatment protocol as outlined in Study 3. Equilibrium moduli of HA constructs increased from 1.74 ± 6.91 on day 0 to 19.24 ± 6.08 kPa after 21 days in CM+ to 44.83 ± 7.4 kPa after an additional 21 days in CM+. Transfer to CM- at day 21 led to a modulus of 32.52 ± 5.14 kPa. Loading of constructs in CM+ conditions over 21 days resulted in modulus values of 37.72 ± 6.68 kPa for 3 Hz, 37.86 ± 10.74 kPa for 1 Hz, and 38.71 ± 7.22 kPa for 0.33 Hz. Long term loading of constructs in CM- conditions resulted in a more dramatic loss of mechanical properties, with 3 Hz, 1 Hz, and 0.33 Hz loaded constructs exhibiting equilibrium modulus values of 14.7 ± 2.94, 18.71 ± 2.47, and 21.64 ± 5.67 respectively.

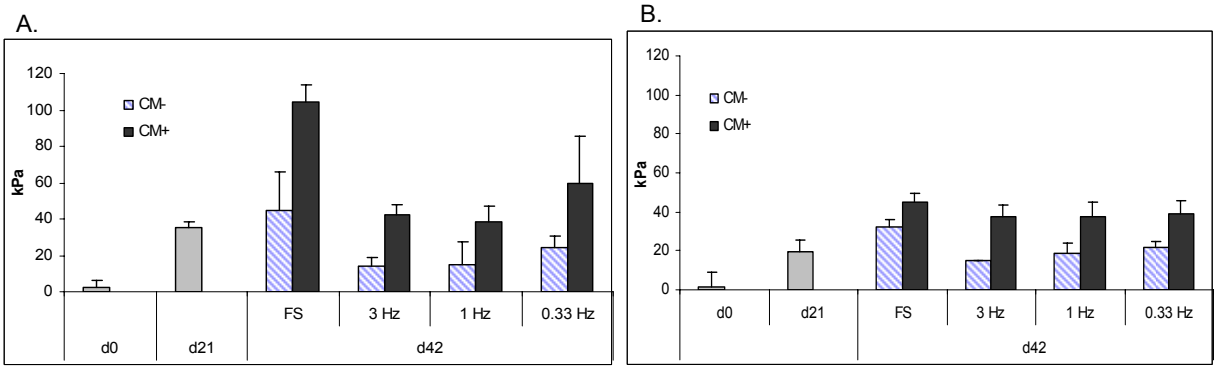
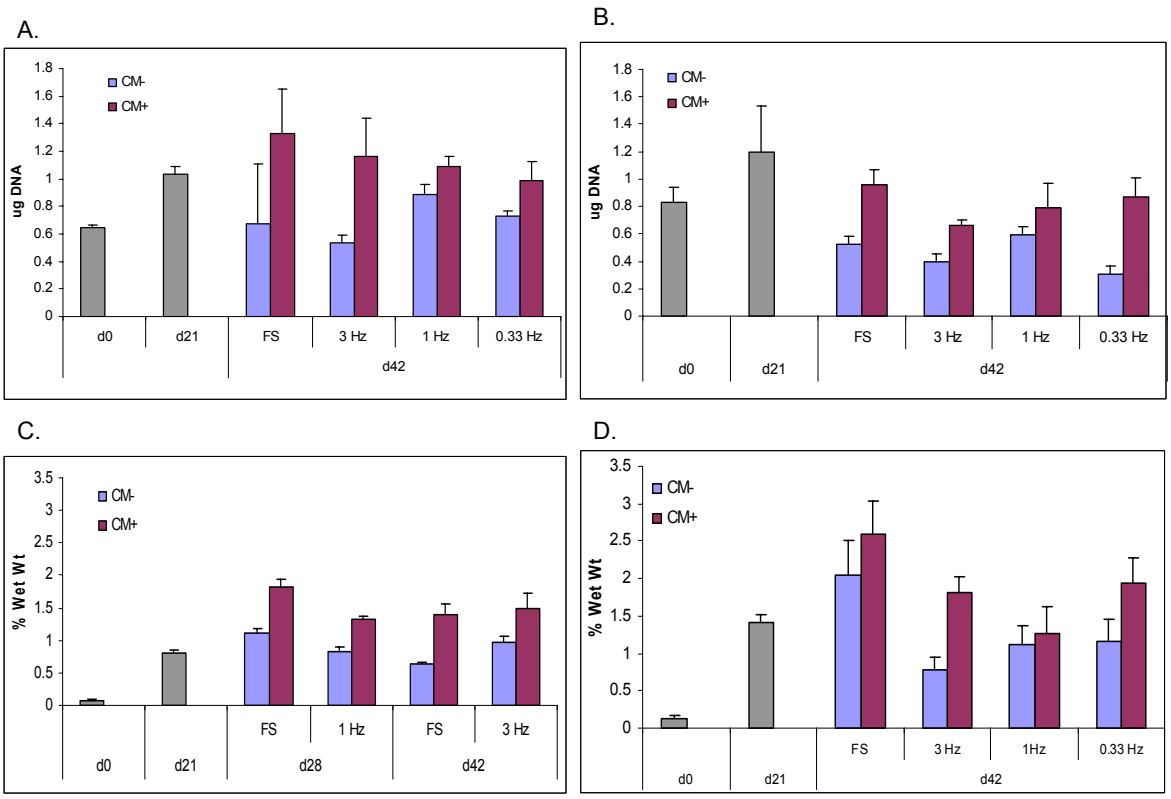


Fig 12. Equilibirum modulus values of A) agarose and B) HA constructs show that prolonged dynamic loading has a detrimental effect on construct mechanical properties regardless of frequency (n = 4)

In agarose constructs, equilibrium modulus values rose from 2.21 ± 4.34 kPa at day 0 to 35.17 ± 3.62 kPa at day 21 to 103.95 ± 9.44 kPa after an additional 21 days in CM+. Transfer of constructs to CM- conditions resulted in modulus values of 44.39 ± 21.4 kPa. Long term dynamic loading in CM- conditions resulted in a decrease in modulus values to 37.86 ± 10.74 kPa, 37.86 ± 10.74 kPa, and 37.86 ± 10.74 kPa for 3, 1, and 0.33 Hz respectively. Similarly, dynamic loading in CM+ conditions resulted in decreased modulus values of 37.86 ± 10.74, 37.86 ± 10.74, and 37.86 ± 10.74 for 3, 1, and 0.33 Hz respectively.

3.5 Biochemical Analysis of Long Term Loading Effects on MSC-Laden Agarose and HA Constructs

Biochemical content for agarose and HA constructs were comparable to the mechanical properties of the constructs. Total DNA content per construct increased over 42 days of free swelling culture in HA hydrogels, indicating MSC proliferation in three dimensional hydrogel culture. Similar changes in DNA content were observed in agarose hydrogels. Long term dynamic loading did not significantly affect DNA content of either agarose or HA constructs in CM+ conditions. However, transfer of constructs into CM- led to a dramatic decrease in DNA content in both hydrogel systems.



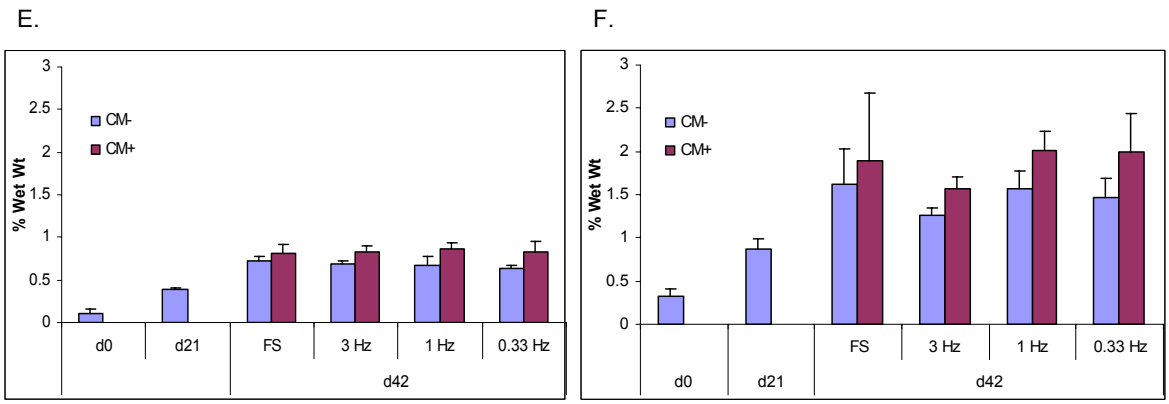


Fig 13. Biochemical properties of MSC-laden hydrogels. DNA content of A) HA and B) agarose constructs, proteoglycan content of C) HA and D) agarose constructs, and total collagen content of E) HA and F) agarose constructs (n = 4)

Agarose constructs also exhibited significantly decreased GAG biosynthesis in response to long term dynamic loading. Staining of agarose constructs with Alcian blue for proteoglycan content shows steady increases GAG content with over time in free swelling CM+ culture. Constructs transferred to CM- conditions showed a noticeable loss in Alcian blue staining. These observations are consistent with GAG content as quantified using 1,9-dimethylmethylene blue dye binding assay. GAG wet weight percentage peaked at 2.59 ± 0.45 % after 42 days of free swelling CM+ culture, while removal of the TGF- β3 chondrogenic signal decreased wet weight percentage to 2.05 ± 0.46 %. In addition, long-term dynamic loading at all frequencies decreased GAG biosynthesis in CM- and CM+ levels. Less marked decreases in GAG biosynthesis were observed in the HA hydrogel systems as a result of dynamic loading. GAG content decreased from 1.81 ± 0.17 % in free swelling CM+ conditions at d42 to 1.39 ± 0.22 % in CM+ at 1 Hz dynamic loading. In both hydrogel systems, transfer of constructs to CM- conditions after 21 days led to noticeably decreased rates of GAG biosynthesis regardless of mechanical environment.

Total collagen content of both agarose and HA constructs increased with 21 days in CM+ media. Constructs continued to deposit collagen even after being transferred in CM- although rate of collagen deposition decreased. Collagen deposition in agarose constructs was more robust than in HA constructs in all conditions and at all time points, although HA constructs showed smaller variations in collagen content.

4. Discussion and Conclusions

In this series of studies, we have used both long and short term loading on a novel hydrogel system in order to investigate the effects of dynamic deformational loading on changes in gene expression and matrix deposition rates of MSCs. Our results suggest that genes controlling ECM biosynthesis – GalNAc, C4st-1, C4st-2, and XT-1 – are mechanically sensitive to dynamic deformational loading. In C4st-1, C4st-2, and XT-1, mechanical loading has a synergistic effect with the chemical morphogen TGF- β3 over the short term, leading to dramatic increases in gene expression over the short term (Fig. 8A, 8B, 9A, 9B). In contrast, TGF- β3 exposure seems to have an antagonistic effect on GalNAc expression, although short term dynamic loading nonetheless increases GalNAc expression both in the presence and absence of TGF- β3 (Fig. 8C, 8D). Moreover, a comparison between short and long term gene expression results would

suggest that general changes in gene expression would occur approximately 24 hours after induction of either a chemical or mechanical signal. This evidence is supported by the fact that with the exception of GalNAc, short term gene expression levels are comparable to and in some cases more robust than long term gene expression levels in corresponding conditions (Fig. 10A, 10B, 10C, 10D).

While upregulation of all matrix biosynthesis genes in response to dynamic loading would seem to imply an improvement in long term mechanical properties in response to long term loading, the opposite is actually observed. Long term loading of constructs resulted in a unilateral decrease in mechanical properties across all frequencies (Fig 12A, 12B). This loss of mechanical properties is especially exacerbated by TGF- β3 removal (Fig 12A, 12B). Similar decreases can be observed in proteoglycan content in response to loading and TGF- β3 removal (Fig. 13A, 13B). Unexpectedly, MSCs cultured in free swelling conditions did not experience a decrease in mechanical properties upon removal of TGF- β3, although rates of increase in mechanical properties decreased in comparison to continuous CM+ culture. Moreover, quantification of DNA content within constructs showed that dynamic loading did not significantly inhibit cell proliferation (Fig 13E, 13F). This indicates that the decrease in mechanical properties and matrix proteoglycan content was due to decreased matrix biosynthesis as opposed to decreased cell numbers between loaded and non-loaded constructs. A number of possible explanations exist for these observed results. The first of these is that dynamic loading simultaneously upregulates both anabolic and catabolic genes. In this scenario, catabolic effects from enzymes responsible for matrix breakdown, such as matrix metalloproteinases, would supersede the anabolic effects caused by matrix biosynthesis genes. Alternatively, it is likely that the long-term loading regime applied in this series of studies is not optimal for matrix deposition in MSCs. In this series of studies, we applied a 10% deformational for 3 hours per day for three alternating days every week. While such a loading regime has been determined to be optimal for ECM production in chondrocyte-laden agarose constructs, the strength and duration of these mechanical signals may not translate to optimal matrix production in MSCs.

Interestingly, we observed marked differences in levels of gene expression between HA and agarose hydrogels. In Study 1, we showed that HA hydrogels are capable of sustaining MSC growth and chondrogenesis at levels comparable to that of agarose hydrogels (Fig. 6, 7A, 7B). In Study 3, comparison in long term gene expression levels showed that all genes are upregulated in HA in significantly higher levels than in agarose. The most marked differences exist for GalNAc, for which expression levels in HA were approximately five times that of agarose. In addition, mechanical and biochemical analysis suggests that HA constructs did not experience as dramatic a drop-off in modulus or proteoglycan content when loaded in CM- conditions. These results could be attributed to the presence of cell-binding ligands on the HA hydrogel not found in agarose that facilitate signal transduction.

5. Recommendations

While the data outlined in these studies seem to indicate that gene expression is upregulated by dynamic deformational loading, more replicates are required to confirm this finding. Additional replicates are also required of the long term dynamic loading studies in order to confirm changes in matrix biochemical content. The studies as outlined in this paper were meant to address a

multitude of variables simultaneously, leading to a large number of conditions and samples. Dividing Study 3 into a series of smaller studies may be beneficial in further clarifying the exact relationship between mechanical and chemical signaling environments.

In addition, future studies will elucidate the factor or factors that prevent gene expression from being translated to matrix biosynthesis. The fact that MSCs have shown the ability to upregulate matrix biosynthetic genes in response to dynamic loading implies the existence of a loading regime that optimizes ECM biosynthesis. Mauck et. al. demonstrated that MSCs increased proteoglycan deposition in long term free swelling culture after a single 180 minute loading cycle at 1 Hz. Coupled with the results from the studies outlined in this paper, this finding suggests that allowing long-term loaded constructs to culture extensively in free swelling conditions would perhaps yield a similar increase in matrix deposition. In addition, long term gene expression results from Study 3 suggest that HA enhances the transduction of extracellular mechanical signals when compared to agarose. Examining short term signal transduction in HA hydrogels via Western Blotting would clarify mechanotransduction in MSCs.

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