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Mechanical Loading and RGD-Conjugation: Influences on Mesenchymal Stem Cell Response

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Introduction

Mechanobiology is an interdisciplinary field that investigates the influence of physical forces and environment on biological processes at cellular and molecular levels. This field is critical for understanding cellular perception, modification, and response to mechanical stimuli, impacting cellular architecture, signaling, and function. Forces such as shear stress, tension and compression are pivotal in regulating cellular behaviors like growth, migration, and differentiation [1].

Mechanotransduction describes the process by which cells convert mechanical stimuli into biochemical signals, a fundamental aspect of cellular functionality. This conversion allows cells to adapt to their physical surroundings by altering gene expression, protein synthesis, and overall behavior. Through components like integrins, ion channels, and the cytoskeleton, mechanotransduction facilitates dynamic interactions between cells and their environments. In stem cell biology, these mechanical cues are essential for directing stem cell differentiation, influencing proliferative capacity, and controlling migration, crucial for tissue repair and homeostasis [2], [3].

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of differentiating into a variety of cell types, including osteoblasts, chondrocytes, and adipocytes. These cells can be isolated from various tissues, notably bone marrow and adipose tissue, making them highly valuable for regenerative medicine and tissue engineering applications due to their accessibility and differentiation capabilities [4]. Mechanical loading is a critical factor in MSC research because it simulates the biomechanical stresses that MSCs experience in their natural tissue environments. This simulation is essential for understanding how MSCs respond to physical cues, which is key to optimizing their therapeutic potential for tissue repair and regeneration.
Figure 1: primary mechanosensitive signaling pathways in MSCs. Figure modified from Saidova et al., 2020.

Figure 1 details a complex array of cellular signaling pathways critical to the mechanotransduction processes in MSCs. These pathways enable MSCs to sense and respond to physical and chemical cues in their environment. Central to this network are the growth factor receptor signaling pathways, which begin with the phosphorylation of receptors by growth factor proteins, triggering a sequence of downstream events. Proteins such as GRB2 and p85 are recruited to activate SOS, which then catalyzes the activation of Ras, switching it from its GDP-bound state to GTP-bound. This activation leads to the initiation of further signaling cascades like the PI3K-Akt, crucial for regulating cell survival and growth [5].
Another critical component shown in Figure 1, involves the phosphorylation of YAP and TAZ by LATS (activated by MST1/2) that acts to keep these transcriptional regulators in the cytoplasm, thereby inhibiting their transcriptional activity in the nucleus that can drive fibroblastic differentiation and cell phenotype changes [6]. Additionally, cell-matrix signaling plays a significant role in cell-matrix adhesion, indirectly influencing RhoA activity and pathways involving YAP/TAZ, essential for cellular responses to the extracellular matrix [7]. These pathways may be dependent on mechanisms that enhance cell adhesion including cellular interactions with extracellular matrix proteins through integrin receptors [8]. Numerous studies have sought to reveal specific features of matrix-integrin interactions by studying cell responses to matrix-derived short peptides, such as RGD that is a component of fibronectin [9]. Understanding select components of these signaling networks is crucial for the development of biomaterials and mechanical loading regimes that enhance MSC therapeutic efficacy, ultimately improving regenerative medicine outcomes and facilitating tissue repair and functional recovery.

This study investigates the variable responses of MSCs from bone marrow versus adipose tissue under mechanical loading within alginate matrices modified with cyclic RGD (cRGD) peptides. We explored the dynamic expression of inflammation and stress-related genes and monitored nitric oxide secretion. The work described in this independent study report is ongoing and the results presented here are preliminary findings. By integrating gene expression analysis with secretion measurements, this research will investigate whether cRGD conjugation may offer protective effects against adverse environments such as injurious compression.
Materials and Methods

Materials and methods were provided by Professor Nathaniel Huebsch and Sydney Neal.

High G-block Manugel alginate (Dupont, Wilmington, DE, USA) was prepared as a 1% (w/v) solution in Dulbecco’s PBS (dPBS). The solution underwent dialysis against deionized water for purification and was sterilized through a 0.22-μm filter under aseptic conditions. The purified alginate solution was subsequently freeze-dried and stored at -80°C.

Cyclic Arg-Gly-Asp (cRGD) peptides were attached to alginate using strain-promoted azide-alkyne cycloaddition (SPAAC) click chemistry, as previously detailed [10]. Briefly, alginate was dissolved in 0.1 M MES buffer (pH 6) and stirred for two hours. Following this, a EDC (Thermo Scientific), NHS (Fisher Scientific), BCN-amine (Sigma-Aldrich, St. Louis, MO, USA) solution was made. This mixture was allowed to react for 24 hours at room temperature. Afterward, the BCN-modified alginate was precipitated, dried, redissolved, frozen, and freeze-dried. The BCN-modified alginate was mixed in dPBS with azide-modified cRGD peptides, stirred for 24 hours at room temperature to reach a peptide concentration of 50μM. It was subsequently purified by dialysis against deionized water, sterilized using a 0.22-μm filter, freeze-dried, and stored at -80°C for later use.

Bone marrow-derived MSCs (RoosterBio™, Frederick, MD) or adipose tissue derived MSCs (ATCC®, PCS-500-011™, Manassas, VA) were encapsulated at a density of 2.5×10^6 cells/mL in 2% (wt/v) alginate either with or without cRGD conjugation, achieving a final polymer concentration of 1% (wt/v) as outlined in previous studies [11]. The polymer solution containing the cells was rapidly mixed with 100mM calcium sulfate and then evenly spread between two sterile glass plates separated by 1.5 mm spacers. The mixture was left to crosslink for one hour under ambient conditions in a cell culture hood. Subsequently, a sterile 6mm diameter biopsy
punch was employed to shape the hydrogel into cylindrical discs, each with a height of 1.5 mm and a diameter of 6 mm. For BM-MSCs, after formation, the hydrogel discs were initially cultured in RoosterBio™ basal medium enhanced with RoosterBio™ booster for six hours. For the rest of the study period, the hydrogel constructs were maintained in RoosterBio™ basal medium that lacks the serum components of the booster media. For A-MSCs, encapsulated cells were cultured with MSC basal medium (ATCC®, PCS-500-030™, Manassas, VA) and growth kit (ATCC®, PCS-500-040™, Manassas, VA). For the first 6 hours after encapsulation, medium was supplemented with 2% FBS and then removed and cultured without FBS for the remainder of the study. All media throughout the study was supplemented with 1% penicillin-streptomycin.

Mechanical loading was applied using a custom 24-well bioreactor as described previously [12]. The components and configuration of the well compression setup are illustrated in Figure 3. Constructs underwent cyclic compression with a trapezoidal waveform, characterized by a 5% static strain offset, an 8% dynamic strain amplitude, and a frequency of 0.33 Hz. Loading was applied for one hour every day for three days. Figure 2 depicts the experimental timeline. After compression, constructs were allowed to free swell in media until the next loading cycle.

**Figure 2:** Experimental timeline. After encapsulation, loading was performed at every 24-hour increment for one hour. First timepoint is immediately after the first loading cycle; final timepoint happens immediately after the final loading.
Nitrite production was quantified in culture media collected after the first and third loading cycles using the Griess assay (Promega, Madison, WI). For RT-PCR analysis, five cRGD-alginate and five alginate gels were collected after the first and third loading cycle and pooled. RT-PCR was performed as described previously [11]. PCR primers were used for interleukin 6 (IL-6), cyclooxygenase 1 (COX-1) and 2 (COX-2), and prostaglandin E synthase 2 (PTGES-2). The concentration of nitrites is reported as mean ± SD for at least eight independent samples per condition. PCR results are reported as the average ΔΔCt for n=2 technical replicates of the pooled gels. Comparisons were drawn between alginate and cRGD-alginate at two timepoints (24 hr and 72 hr) to assess the impact of mechanical loading and the potential protective role of cRGD against oxidative stress in MSCs.

**Figure 3:** a) schematic of the mechanical loading system, detailing each component. b) Plastic platen. c) Hydrogel. d) Porous PDMS insert designed for hydrogel confinement and nutrient diffusion. e) Solid PDMS insert that supports the porous insert and forms the base for the hydrogel. f) Individual well of a 24-well plate.
Results and Discussion

Exploring MSCs from different sources, such as bone marrow versus adipose tissue, is crucial for corroborating proposed regenerative medicine strategies. Each source offers distinct advantages in terms of cell availability, proliferative capacity, differentiation potential, and immunomodulatory properties. For instance, BM-MSCs are traditionally noted for their robust osteogenic potential, making them ideal for bone and cartilage repair [13]. In contrast, A-MSCs are more abundantly available and easier to harvest with less invasive procedures, providing a practical advantage for therapeutic use [13]. Additionally, different MSC sources may exhibit varied responses to inflammation and possess unique secretory profiles, affecting their therapeutic efficacy in treating diseases [14]. By comparing MSCs from these diverse origins, cell therapy approaches can be better tailored to specific clinical needs, enhancing treatment effectiveness, and broadening the scope of applications in tissue engineering and regenerative therapies.

Figure 4 shows the resulting fold change of selected genes at the two timepoints explored for BM-MSCs, Figure 4a-d, and A-MSCs, Figure 4e-h. For both A-MSCs and BM-MSCs, there is a consistent increase in IL-6 expression from 24 to 72 hours in both alginate and cRGD conditions, suggesting an ongoing inflammatory or stress response within the MSCs. IL-6 is a pro-inflammatory cytokine, often upregulated under stress conditions such as mechanical loading or inflammatory stimuli. For BM-MSCs, COX-1 expression shows a slight decrease in the cRGD group from 24 to 72 hours, and a slight increase in the alginate group; however, both changes are minimal. For A-MSCs, COX-1 expression change is even less pronounced for both groups. Differences at or below 2-fold could be within the limits of error. COX-1 is a constitutive gene and therefore, it would be expected that it would show minimal changes. A marked increase in COX-2 expression is observed in both alginate and cRGD conditions from 24 to 72 hours for BM-MSCs;
interestingly, the cRGD-alginate group shows a slightly higher expression of COX-2. This trend is not observed for A-MSCs, instead there is a greater increase in gene expression for the alginate group versus the cRGD-alginate. COX-2 is an inducible enzyme often associated with the acute phase of inflammation and is typically upregulated in response to mechanical stress and cytokine production [15]. BM-MSCs PTGES-2 expression shows upregulation from 24 to 72 hours in both conditions; expression is very similar for A-MSCs. PTGES-2 plays a crucial role in cellular inflammation and stress management. It is instrumental in the production of prostaglandin E2 (PGE2), a key mediator of inflammatory responses. Nitric oxide synthase 1 (NOS-1) expression was not detectable by PCR for A-MSCs, but not for BM-MSCs; corresponding data can be found in the appendix (Figure A1).

In general, these finding support previous research that loading increases the stress state of encapsulated cells [16]. There was little evidence of differences in the MSC cellular response when embedded in cRGD-alginate versus unmodified alginate. The data suggests cRGD might trend towards mitigating an inflammatory or stress response; however, with the lack of samples, no definitive statements can be made. Moreover, the cells are cultured primarily without serum throughout loading, this condition itself may promote the observed stress and inflammatory response observed over time. Further studies would benefit greatly from both more samples for analysis and a control group of unloaded gels.
Figure 4: Displaying fold change of gene expression for selected genes in a-d) BM-MSCs and e-h) A-MSCs. Data reported calculated from mean (n=2) of ΔΔCt. All of COX-1 is insignificant and COX-2 is borderline. More biological replicates are needed.
Measuring both cell gene expression and protein or metabolite secretion is crucial for a thorough understanding of cellular functions and regulatory mechanisms. Gene expression analysis provides insights into which genes are actively transcribed into mRNA, reflecting the cell’s regulatory processes at the genetic level. However, the levels of mRNA do not always correlate with the concentrations of proteins or other secreted molecules due to factors like post-transcriptional modifications and variable molecule stability. By also measuring the secretion of proteins and molecules, the functional output of the cell, encompassing not just protein functions but also the roles of other key signaling molecules can be assessed. This combined approach enables a more accurate interpretation of how genetic information is manifested in cellular behavior, improving understanding of disease pathways, cellular responses to environmental stimuli, and the development of targeted therapies.

Nitric oxide (NO) is an essential molecule to measure when analyzing cellular behavior, particularly in the contexts of stress and inflammation, due to its versatile roles as a signaling molecule in various physiological and pathological processes. As a free radical with a very short half-life, NO can act locally to mediate immune responses [17]. In inflammatory conditions, NO production is often upregulated, serving both cytotoxic and cytoprotective functions [18]. Excessive, or insufficient production of NO can be indicative of pathological states. For instance, high levels of NO can contribute to tissue damage and chronic inflammation, while insufficient NO can impair blood flow and wound healing [19]. Thus, measuring NO alongside gene expression and protein secretion provides insight into the health of cells and their responses to stress and inflammatory stimuli, enabling more effective diagnostic and therapeutic strategies.

Nitric oxide production through a Griess assay was analyzed, which gives the nitrite concentration in solution. Nitric oxide itself is a short-lived, correspondingly, its concentration in
solution is difficult to directly assess; however, nitrite is a stable product of nitric oxide and represents an appropriate proxy for cell production. The resulting analysis shows a small increase in nitrite concentration from 24 to 72 hours. There is an increase in nitrite concentration for cRGD-alginate groups compared to naked alginate, however this is a very small increase and not significant.

**Figure 5** depicts the resulting nitrite concentration in medium at each timepoint for each cell for cRGD and alginate. From these results, it appears mechanical loading may increase nitric oxide production; however, a comparison to a non-loaded control is required. Additionally, an analysis of cell death in the hydrogel would be beneficial. If cRGD provides any protective effect against adverse environments, it would allow cells to release metabolites for longer, increasing concentration. Previous studies have demonstrated growth factors reducing nitrite concentration after 3D encapsulation [20]. An analysis of how these growth factors may interact with both adhesion pathways and oxidative phosphorylation would be important for MSC’s future viability in clinical applications.

**Figure 5**: Medium nitrite concentration for a) BM-MSCs b) A-MSCs
Conclusion

This report summarizes a preliminary study that sought to assess the differential responses of MSCs derived from bone marrow and adipose tissue when encapsulated with cRGD conjugated to the extracellular matrix and subject to dynamic mechanical loading. We focused on the dynamic expression of inflammation and stress-related genes such as IL6, COX1, COX2, and PTGES2. Our results demonstrate a consistent increase in IL6 expression over time in both MSC types, suggesting an ongoing inflammatory or stress response, with a more pronounced increase observed in A-MSCs. cRGD conjugation slightly reduced IL6 expression, though the limited sample size precludes definitive conclusions.

COX-1 levels remained stable across all conditions, consistent with its constitutive nature. In contrast, COX-2 expression, which is inducible, slightly increased in BM-MSCs encapsulated in cRGD-alginate compared to naked alginate. This trend was reversed in A-MSCs, where cRGD conjugation decreased COX2 expression. PTGES-2 expression generally increased in both cell types with mechanical loading, but showed attenuation in cRGD-alginate conditions compared to the unmodified alginate. Additionally, the Griess assay results revealed a modest increase in nitrite levels over time, particularly in cRGD-alginate groups. This increase does not support the hypothesis that cRGD may have protective effects against adverse loading environments; however, the interplay between cell adhesion and stress/inflammation is still an important area of concern. There is some evidence for the cross-talk between adhesion and growth-factor signaling pathways, more research into how this may be leveraged and the exact nature of interaction is needed.

A significant limitation of this study was the lack of replicates in PCR analysis, which restricts the robustness of our findings. The absence of control groups, particularly a non-loading control, leaves unanswered questions about the impact of serum deprivation on observed stress
effects. Given the unclear differences between the expression of specific genes like IL-6 and COX-2 among the cell sources, further investigation into gene expression and secretion profiles is essential to elucidate their viability for regenerative medicine applications. Indeed, work is ongoing to increase biological sample number and to include appropriate control groups to address the major questions about mechanical loading on MSCs.

Future studies would greatly benefit from larger sample sizes and the inclusion of control groups to better understand the potential of MSCs in regenerative medicine. This research highlights the importance of exploring the combinatorial effects of mechanical loading and cell-matrix interactions on MSC functionality.
Appendix

Figure A1 shows the fold change gene expression for nitric oxide synthase 1 (NOS-1) for adipose derived MSCs. NOS-2 was also targeted but was undetectable in either cell type. Interestingly, NOS-1 was undetectable in BM-MSCs. The implications of this are not particularly clear. As observed in Figure 4, gene expression is similar across both cell sources, it would be unexpected for NOS-1 to be too low to be measured in only BM-MSCs. Additionally, Griess shows a higher concentration of nitrite in all conditions for BM-MSCs versus A-MSCs. This indicates a higher level of secretion of nitric oxide despite seemingly no NOS-1 or NOS-2 expression. It is possible that nitric oxide may be contributing to other processes in A-MSCs and not in BM-MSCs given its medium nitrite concentration increase with lower NOS-1 expression.

![Figure A1: NOS-1 fold change for A-MSCs.](image-url)
References


