A New Paradigm for Mechanical Stimulation of 3D Cellular Scaffolds in Tissue Engineering

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INTRODUCTION: Mechanical loading plays a key role in the remodeling and repair of soft tissue, yet the physical mechanism that governs this mechanobiological response under complex *in-vivo* loads remains unknown. This knowledge gap has hindered the development of more effective therapies for soft tissue injuries that are responsible for 15 million hospital visits every year in the U.S.[1] Efforts to understand this mechanism have generally used bioreactors to apply *in vitro* stimulations based on material stress or strain, but these 2nd order tensor quantities possess multiple components that hinder the formulation of a singular theory to predict cellular response under various loading conditions. One intriguing theory is that cell mechanosensing is regulated by material strain energy density (SED).[2] Unlike stress and strain, SED is a unique scalar measure of deformation and can be decomposed into hydrostatic SED, responsible for volume change, and distortion SED, responsible for shape change. SED has yielded theories reliable in predicting structural changes in natural and engineered materials[3] and may generate a unifying theory to predict cell responses to any loads. However, validation of this theory requires automated and accurate experimental methods to apply targeted SED levels under complex loads, and these methods have previously not existed. The objective of this work was to develop a novel automated method to apply targeted SED levels to 3D constructs undergoing dynamic mechanical stimulation.

METHODS: *Bioreactor Hardware and Software.* We designed and built a multiaxial bioreactor that simultaneously applies tensile and compressive loads to cell-seeded constructs (**Fig. 1**). Briefly, it consists of a tissue chamber, two electromagnetic actuators for motion and two 1.0-N force sensors. We then developed LabVIEW programs that apply a user-specified SED under single- or multi-axial cyclic stimulation by using the secant algorithm to iteratively optimize a force input. *Verification of SED Application*. Polyurethane scaffolds (DSM Biomed) were prepared, clamped inside the tissue chamber of the bioreactor, and subjected to one of three loading conditions (n=3/group): uniaxial tension (T), uniaxial compression (C), and biaxial tension-compression at equal magnitudes (TC). A single targeted SED of 100 J/m^3 was prescribed to the samples in all loading cases for 30 minutes at a 1Hz-frequency. The steady state SED was computed and compared to the target value. Additionally, hydrostatic and distortion SED were computed using our previously validated numerical method. A one-way ANOVA and Tukey-HSD post-hoc were performed to detect any differences in energies between loading configurations. *Application to Cellular Bioscaffolds*. To verify that our method did not accelerate cell death, we applied it to cell-seeded collagen scaffolds that were subjected to the TC loading condition. Briefly, dumbbell-cut collagen foams (DSM Biomed) were sterilized, seeded with 3T3 murine fibroblasts (10^6/scaffold, Sigma Aldrich), and statically cultured for 24 h inside an incubator. Our new method was applied to dynamically stimulate the constructs at a 0.25-Hz frequency, for 8 h per day, for 2 days, and at a targeted SED of 100 J/m^3. A free-swelling (FS) group of samples stayed unstimulated inside the incubator for the same period to serve as controls. Cell viability was assessed at the end of the 3-day culture period using an XTT cell proliferation assay (Cayman), which evaluates cell metabolic activity, and confocal microscopy (Zeiss) to visualize cell nuclei (stained with propidium iodide) and actin cytoskeleton (stained with phalloidin) and quantify cell density and proliferation. Independent samples T-tests were performed to detect any differences in XTT absorbance and volumetric cell density values between FS and TC groups (n=6/group).

RESULTS: Our novel method successfully applied the 100 J/m^3 target SED with less than 5% error for all cases ($p = 0.61$). An average of six optimization iterations were required to achieve convergence and hit the targeted SED. When decoupling the energy, the hydrostatic and distortion SED in the TC loading case were significantly different than the T and C loading cases (**Fig. 2**). When applied to the cell-seeded collagen samples, TC loading increased cell metabolic activity in the constructs (**Fig. 3A**) although the cell density values were not significantly different between the TC and FS groups (**Fig. 3B**).

DISCUSSION: This study has developed and verified a novel automated method to apply targeted levels of SED to 3D cellular constructs. For the first time, we accurately applied user-specified SED to acellular constructs that were subjected to distinct loading conditions (uniaxial and planar) with significantly different distortion SED values. The ability to apply targeted amounts of SED under different loading conditions is an important advance, as we can now isolate the effect of distortion and hydrostatic energy on cell behavior. Moreover, the method did not accelerate cell death in the collagen scaffolds during the 3-day culture period. In fact, the TC stimulation significantly increased cell metabolic activity compared to free swelling. Interestingly, ligament, tendon, and other soft tissues experience TC loading conditions *in-vivo* and thereby are physiologically exposed to high levels of distortion energy. The results from this study support strain energy-based stimulation as a feasible and attractive alternative to the standard stress- and strain-based simulation of cellular constructs.

SIGNIFICANCE/CLINICAL RELEVANCE: This new method will finally allow the investigation of new energy-based theories to predict cell response to any loading configuration, which may result in the development of more effective therapies for soft tissue injuries.

REFERENCES: 1. Herzog, L et al, *Orth J Sports Med*, 2013; 2. Panzetta, V et al, *PNAS*, 2019; 3. Von Mises, R, *News from Societies of Sciences*, 1913.

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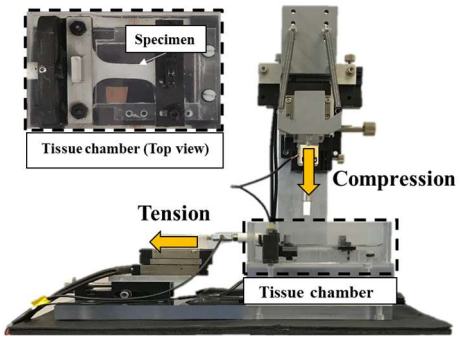

Fig. 1: Custom Tension-compression multiaxial bioreactor

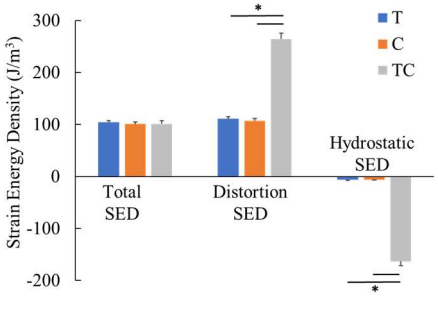

Fig. 2: Total, distortion, and hydrostatic SED in polyurethane samples (* p<0.05)

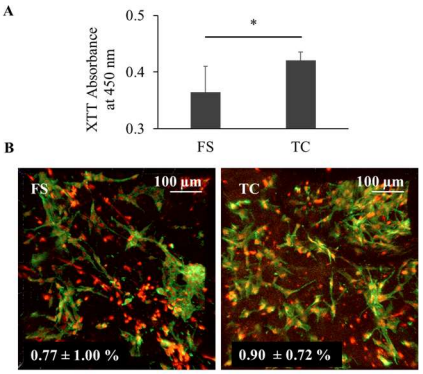

Fig. 3: A) Significant increase in cell metabolic activity from FS to TC. B) Confocal imaging of cell nuclei (red) and actin (green) in collagen.