Video Article

Mechanical Stimulation of Chondrocyte-agarose Hydrogels

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Abstract

Articular cartilage suffers from a limited repair capacity when damaged by mechanical insult or degraded by disease, such as osteoarthritis. To remedy this deficiency, several medical interventions have been developed. One such method is to resurface the damaged area with tissue-engineered cartilage; however, the engineered tissue typically lacks the biochemical properties and durability of native cartilage, questioning its long-term survivability. This limits the application of cartilage tissue engineering to the repair of small focal defects, relying on the surrounding tissue to protect the implanted material. To improve the properties of the developed tissue, mechanical stimulation is a popular method utilized to enhance the synthesis of cartilaginous extracellular matrix as well as the resultant mechanical properties of the engineered tissue. Mechanical stimulation applies forces to the tissue constructs analogous to those experienced *in vivo*. This is based on the premise that the mechanical environment, in part, regulates the development and maintenance of native tissue[1,2]. The most commonly applied form of mechanical stimulation in cartilage tissue engineering is dynamic compression at physiologic strains of approximately 5-20% at a frequency of 1 Hz[1,3]. Several studies have investigated the effects of dynamic compression and have shown it to have a positive effect on chondrocyte metabolism and biosynthesis, ultimately affecting the functional properties of the developed tissue[4-8]. In this paper, we illustrate the method to mechanically stimulate chondrocyte-agarose hydrogel constructs under dynamic compression and analyze changes in biosynthesis through biochemical and radioisotope assays. This method can also be readily modified to assess any potentially induced changes in cellular response as a result of mechanical stimuli.

Video Link

The video component of this article can be found at http://www.jove.com/video/4229/

Protocol

1. Isolation of Primary Articular Chondrocytes

Harvest 10-15 full thickness cartilage slices from the articular surfaces of animal joints (*e.g.* the metacarpal-phalangeal joint of skeletally mature cows obtained from a local abbatoir).

1. Place cartilage slices in a 100 mm Petri dish and Incubate in 20 ml of 0.5% protease in Ham's F-12 (w/v) for 2 hr at 37 °C. Rinse three times in Ham's F-12 culture media, and incubate with 20 ml of 0.15% Collagenase A in Ham's F-12 culture media overnight at 37 °C.
2. Filter the cell suspension through a 200 mesh screen filter into a clean dish. Wash the filter with 5 ml of Ham's F-12 and add to the dish. Transfer the cell suspension to a 50 ml conical tube. Wash the Petri dish with 10 ml of Ham's F-12 and add to the conical tube.
3. Centrifuge the tube at 600-800 rcf for 6-8 min at room temperature.
4. Aspirate the supernatant, ensuring not to disturb the cell pellet and re-suspend in 40 ml of Ham's F-12 culture media.
5. Centrifuge the tube at 600-800 rcf for 6-8 min.
6. Repeat steps 1.4 and 1.5 twice more for a total of 3 times. Prior to centrifuging for the third time, resuspend the pellet via agitation and obtain a 500 µl aliquot for cell counting.
7. Count viable cells using the Trypan blue exclusion method[9] with a hemacytometer and an inverted light microscope.
8. Aspirate the supernatant and re-suspend the pellet in a volume of Ham's F-12 media to double the desired seeding density (*e.g.* 20 x 10^6 cells/ml for a final concentration of 10 x 10^6 cells/ml after encapsulation).

2. Chondrocyte-agarose Hydrogel Encapsulation

1. Heat 0.8 g autoclaved Type VII agarose in 20 ml of sterile 1x PBS (pH 7.4) on a hot plate set to 120 °C until dissolved. Once dissolved, lower the heat to 60 °C as higher temperatures will affect cell viability.

2. In a 50 ml conical tube, thoroughly mix equal volumes of the cell suspension with double the desired concentration of agarose (*e.g.* 4% agarose suspension for a final 2% agarose hydrogel). Avoid the creation of large bubbles as they can affect cell viability and construct fatigue life.
3. Carefully aliquot 10 ml of the chondrocyte-agarose mixture into a 60 mm diameter Petri dish, while avoiding the creation of large bubbles.
4. Let stand for 30 min at room temperature until gelled.
5. Obtain as many chondrocyte-agarose hydrogel samples as needed (typically 20-30) using a 4 mm biopsy punch.
6. Measure and record the physical dimensions (diameter and height) of representative samples using a micrometer. Constructs should be approximately 5 mm in height, discard any samples obtained from a region where the sample height variance was greater than 2%.
7. Transfer samples to a fresh 60 mm Petri dish and supplement with 10 ml of complete media (*e.g.* 20% fetal bovine serum in Ham's F-12 culture media with 20 mM HEPES, 100 µg/ml ascorbic acid, and 2x antibiotics/antimycotics).

3. Mechanical Stimulation and Radiolabelling of Chondrocyte-agarose Hydrogels

1. Autoclave the metallic components of the compression rig. Sterilize the plastic components with 70% ethanol (overnight) and UV light (for at least 15 min).
2. To keep constructs from falling over, using forceps, place 12 sterile plastic retaining rings (with an inner diameter larger than the construct diameter) (n=6, samples and controls) within the wells of a 24-well culture plate.
3. Using forceps, carefully transfer the chondrocyte-agarose hydrogels from the Petri dish to the 24-well plate, securing each one in a retaining ring.
4. Transfer 400 µl of complete media (*e.g.* 20% FBS in Ham's F-12 culture media with 20 mM HEPES, 100 µg/ml ascorbic acid, and 2x antibiotics/antimycotics) to each sample well.
5. Assemble the sterilized compression rig (**Figure 1**) and secure the platens with set screws. Attach the compression rig to the 24-well culture plate.
6. Release and re-secure the set screws to establish platen-hydrogel contact (zero strain state).
7. Load the assembled compression rig into a Mach-1 Micromechanical Testing System. Secure rig to the vertical stage via locating pin and lock in place with set screw. Remove the spacing jig.
8. Apply dynamic compressive loading at desired amplitude (*e.g.* 10% strain amplitude based on the original height of the samples), frequency (*e.g.* 1 Hz) and duration or number of cycles (*e.g.* time intervals up to 60 min).
9. Upon the completion of mechanical stimulation, replace the spacing jig, loosen the locating pin set screw and remove the compression rig and culture plate from the Mach-1 Micromechanical Testing System.
10. Radiolabel cell-hydrogel constructs by supplementing the media with 5 µCi of desired isotope (*e.g.* [^{3}H]-proline protein label for chondrocyte-specific collagen synthesis and [^{35}S]-sulfur for proteoglycan synthesis).
11. Incubate the radiolabeled constructs for 24 hr at 37 °C, 5% CO_2[10].
12. Harvest the radiolabeled hydrogel constructs and rinse 3 times in 1x PBS (pH 7.4) to remove any unincorporated isotope. Digest samples using papain (40 µg/ml in 20 mM ammonium acetate, 1 mM ethyldiaminetetraacetic acid and 2 mM dithiothreitol at 65 °C for 72 hr).
13. Assay digest for DNA content using the PicoGreen dye assay[11] and incorporated isotope activity, normalized to DNA content, by β-liquid scintillation counting[12,13] immediately following papain digestion.

Note: The purchase and disposal of radioactive materials (waste isotopes and items that may have come into contact radioactive materials) must follow the relevant institutional (and/or governmental) policies and procedures for the safe handling and disposal of radioactive substances.

4. Representative Results

Bovine articular chondrocyte-agarose hydrogels constructs (2% agarose with 10 x 10^{6} cells/ml encapsulated cells) were mechanically stimulated at an amplitude of 10% compressive strain at a frequency of 1 Hz for 20 to 60 min (or 1,200 to 3,600 cycles) and assayed for DNA content and extracellular matrix synthesis by radioisotope incorporation. DNA and extracellular matrix synthesis was affected in a dose-dependent manner. DNA content exhibited a significant 35% decrease as a result of 20 or 30 min of stimulation ($p<0.01$), whereas 60 min of stimulation exhibited no effect (**Figure 2**). Cartilage-specific collagen and proteoglycan synthesis (determined by [^{3}H]-proline and [^{35}S]-sulfur incorporation, respectively) exhibited significant increases of approximately 60% in response to 20 or 30 min of stimulation ($p<0.01$), with 60 min of stimulation exhibiting no observable effect (**Figure 3**).

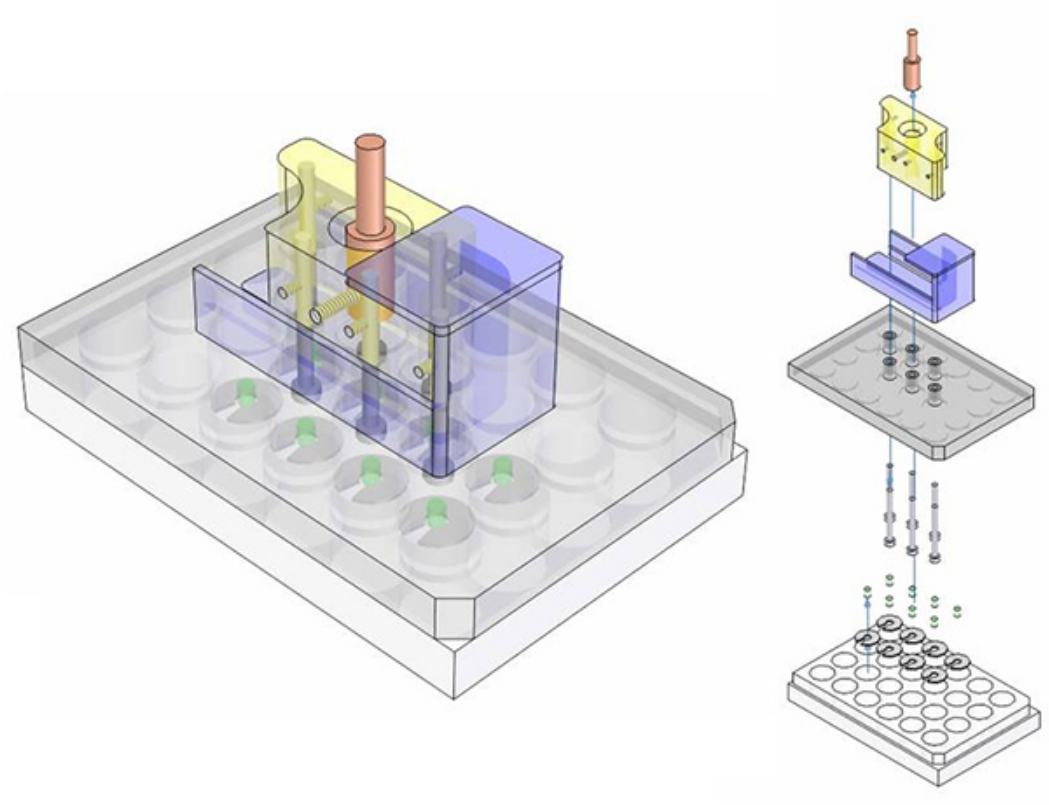

Figure 1. Schematic of the custom-built dynamic compression rig for stimulating chondrocyte-agarose constructs. *Left*: Assembled rig. *Right*: Exploded view showing individual components.

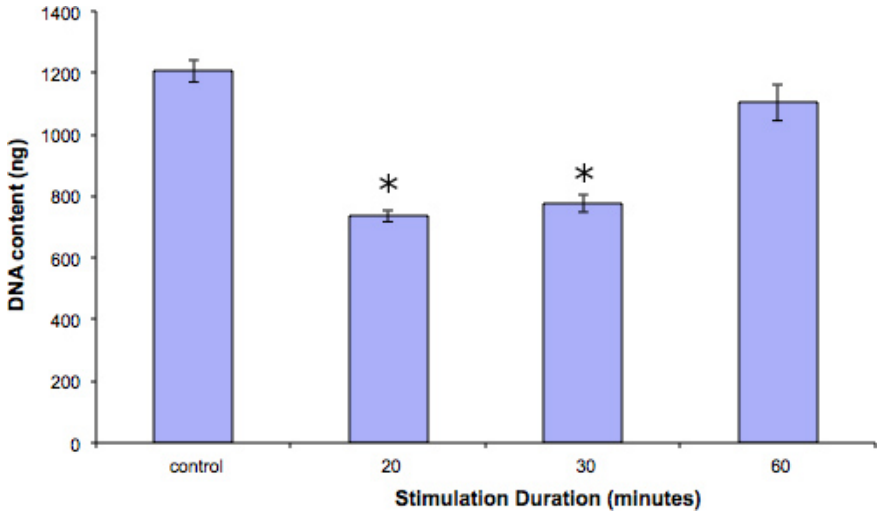

Figure 2. Changes in DNA content of chondrocyte-agarose hydrogels mechanically stimulated under a 10% compressive strain amplitude at 1 Hz for 20 to 60 min (mean ± SEM, n=6/group).

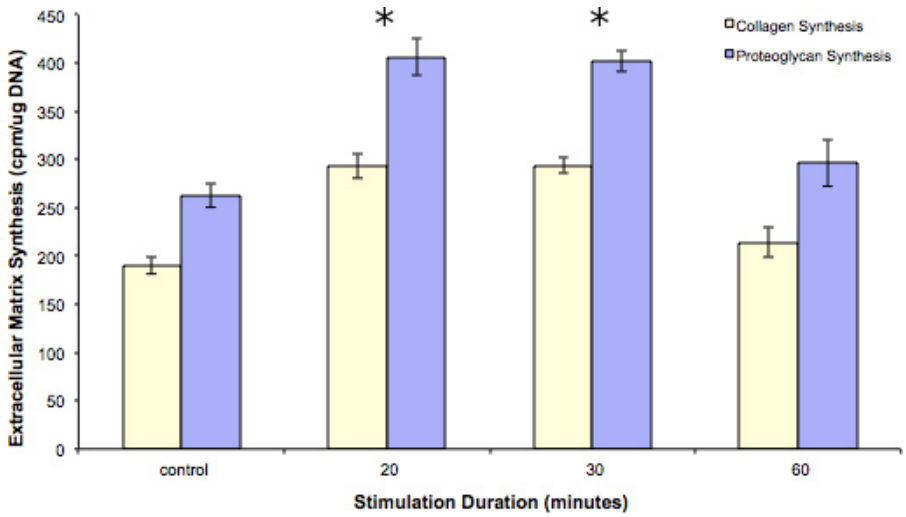

Figure 3. Changes in extracellular matrix synthesis (collagen and proteoglycans) of chondrocyte-agarose hydrogels mechanically stimulated under 10% compressive strain amplitude at 1 Hz for 20 to 60 min (mean ± SEM, n=6/group).

Discussion

The described method for applying controlled mechanical stimuli to cell-seeded agarose hydrogels allows for the direct investigation into the effects of dynamic compressive forces on chondrocyte metabolism. The use of the custom-testing rig in conjunction with the retaining rings provided lateral constraint for the constructs to avoid potential problems of sample tipping. The use of dead-weighted loading platens secured by sets crews ensures direct contact with constructs despite potential differences in sample height. Radiolabeling post-stimulation to measure cellular biosynthesis reduces the potential for contamination, allowing for a single testing rig to be utilized for multiple applications of dynamic mechanical stimuli. This method can be easily adapted to investigate the effect of different mechanical loading modes (*e.g.* shear[1,4], tension[1,2]) or to elucidate specific mechanotransduction pathways responsible.

The representative results illustrated that a small amount of cell death of approximately 35% can occur due to the application of dynamic stimulation, with longer applications of mechanical stimulation not affecting construct cellularity[15-17]. Biosynthesis of extracellular matrix macromolecules, determined by radioisotope incorporation, illustrated a duration dependent response to dynamic compression. Applications of dynamic compressive loading for a period of 20 to 30 min resulted in a maximal anabolic response with longer durations appearing to elicit a desensitization effect to the imposed mechanical stimuli. Cellular desensitization to mechanical loading has been noted previously[1,2,5,13], but there has been little investigation into characterization of the time dependent nature of this phenomena. This information can be used to determine the optimal stimulation conditions to maximize the accumulation of extracellular matrix with the construct under repeated, or long-term, applications of mechanical stimulation.

Disclosures

No conflicts of interest declared.

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