Dynamic Loading, Matrix Maintenance And Cell Injection Therapy Of Human Intervertebral Discs Cultured In A Bioreactor.

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Introduction: Low back pain affects the quality of life for millions of people, and it presents a major problem for direct and indirect global healthcare costs. Intervertebral disc (IVD) degeneration is directly associated with low back pain. Long-term organ culture of human IVDs is essential for developing ex vivo models for study of IVD degeneration and repair. Using this ex vivo approach, the relationship between mechanobiology, disc matrix composition and metabolism can be better understood in the context of degenerative disease. To establish this ex vivo organ culture model, a bioreactor was developed that facilitates organ culture of intact large discs in a controlled dynamically loaded environment. The bioreactor is used in combination with a previously reported harvesting method which maintains the integrity of the intervertebral discs by preserving the non-calcified part of the cartilage endplate with collagen fibers attached to it. Here, we determine IVD tissue integrity and cell viability under 3 different loading conditions, and investigate the suitability of this model towards cell supplementation for tissue repair.

Methods: Disc Isolation and Preparation: Human lumbar IVDs were obtained through organ donations via Transplant Quebec. The spines were assessed by X-ray to evaluate degree of degeneration. Intact discs were prepared by parallel cuts through the adjacent vertebral bodies close to the end plates. All calcified tissue was removed using a high-speed burr to prepare discs with cartilaginous endplate. Pictures of the disc were taken after processing and surface area was calculated with ImageJ software to calculate the load to be applied.

Bioreactor Culture and Loading: IVDs were transferred to the bioreactors and were loaded statically for 48 hours at 0.1 MPa allowing the discs to creep, thereby equilibrating its water content to the external load and intrinsic swelling properties. 21 healthy human discs were divided into three groups: low, medium, and high. They were loaded with dynamic compressive loads cycling in a sinusoidal pattern between 0.1 MPa and 0.3, 0.6 or 1.2 MPa respectively, for two periods of 2 hours each. The dynamic compressive load periods were interrupted by recovery periods of 6 hours and 14 hours respectively, where they maintain a low static 0.1 MPa load. The scheme was repeated for 10 consecutive days.

Cell Viability: After the 10 day loading protocol, portions of nucleus pulposus and annulus fibrosus were excised and analyzed via LIVE/DEAD assay (Invitrogen), and smaller sections were visualized using a laser-scanning confocal microscope. The proportion of viable cells was quantified.

Proteoglycan Content Assessment: Proteins and proteoglycans were extracted on a wet weight per volume basis using 15 volumes extraction buffer (4 M guanidinium chloride, 50 mM sodium acetate, pH 5.8, 10 mM EDTA, with protease inhibitors) at 4°C under continuous agitation for 48 h.
Western Blot: 40 µL aliquots of protein extract were ethanol precipitated, washed, and re-dissolved in 200 µL SDS sample buffer. 20 µL of this was separated by SDS-PAGE and then transferred to nitrocellulose membranes and immunoblotting was performed using antibodies against chondroadherin (CHAD) followed by secondary antibody conjugated with HRP (1:2000 dilution) in blocking buffer containing 1% BSA. Visualization was performed by chemiluminescence using the ImageQuant LAS4000 (GE Healthcare, Baie d’Urfe, Qc, Canada). ImageQuant TL analysis toolbox software (GE Healthcare) was used to analyze the blots.

Cell Injections for Biological Repair: One million primary fluorescently labeled human NP cells were seeded per 1 mL of thermogelling hyaluronic acid hydrogel (HA-pNIPAM). ~250 µL of cell/gel mixtures were injected laterally into the NP region of isolated whole discs (see above). After 48 h of static load (100 N), discs were dynamically loaded with 0.1 - 0.6 MPa load protocol indicated above for 3 days. As a control, one disc remained unloaded for the duration of the experiment. After three days, discs were dissected, and sections of the NP were assessed for localization and viability of the labeled injected cells using a laser scanning confocal microscope.

**Results:** After 14 days in dynamic culture, cell viability was maintained at greater than 80% throughout the disc at low and medium loads. Viability dropped to approximately 60-70% throughout the disc under high loads. Proteoglycan content remained stable in all loading protocols (approximately 50 µg sGAG/mg tissue). No fragmentation of Chondroadherin was observed with the low, medium and high loading regimes. To test for feasibility of cell therapies in the bioreactors, NP cells combined with a hydrogel were injected into whole human discs and cultured under medium load. Three days after dynamic culture, injected human NP cells within an HA-pNIPAM hydrogel showed dispersed nuclear and annular localization with greater than 70% viability.

**Discussion:** This study indicates feasibility of culturing human IVDs for 14 days of various physiological (moderate to strenuous) loads. Our results demonstrate that IVDs maintain cell viability and tissue homeostasis and integrity when cultured under 3 different physiological loads. The isolation technique and culture system provides an avenue towards an ex vivo model system where numerous parameters in addition to load can be evaluated, e.g. the effect of growth factors, O2 tension, glucose levels, and bioactive therapeutics. The bioreactor can also be used to evaluate changes in mechanical properties of the disc following either biologically-induced changes, or used to induce biomechanical stimulus of the disc to generate a biological change. More importantly, this ex vivo model can be used as a platform on which to study cell based therapies, by injecting isolated primary disc cells or stem cells into whole discs. Injected cells can be co-applied within various hydrogels enhancing their reparative properties. When combined with the capabilities of the bioreactor, this ex vivo approach can elucidate the role of load in both disc degeneration and tissue regeneration.

**Significance:** The bioreactor provides an experimental platform useful to evaluate the effect of load alone as well as to determine if biologic repair strategies combining injectable hydrogels and isolated NP cells is feasible over a range of loading conditions. Such knowledge is important for determining the value of cell/hydrogel injections and patient advisement on physical activities following a biological repair procedure.
Figure 1. Effects of dynamic loading on human discs injected with NP cell/HA-pNIPAM gels. A) An isolated human L3/L4 whole disc being injected with fluorescently labeled human NP cells. B) Schematic of the loading scheme applied to the injected disc (repeated over 3 days). Medium load only was applied here. C) Representative confocal images showing labeled (green) and dead cells (red, ethidium homodimer). Green cells are living injected NP cells, red cells represent dead native cells, and yellow cells represent dead injected cells. Scale bar indicates 200 μm. Schematic at bottom indicates the track which the needle made upon injection of cells, while red circles indicate regions where images were acquired for AF and NP regions.