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Novel Ex-vivo Mechanobiological Intervertebral Disc Culture System

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Abstract

Intervertebral disc degeneration, a leading cause of low back pain, poses a significant socioeconomic burden with a broad array of costly treatment options. Mechanical loading is important in disease progression and treatment. Connecting mechanics and biology is critical for determining how loading parameters affect cellular response and matrix homeostasis. A novel ex-vivo experimental platform was developed to facilitate in-situ loading of rabbit functional spinal units (FSUs) with relevant biological outcome measures. The system was designed for motion outside of an incubator and validated for rigid fixation and physiologic environmental conditions. Specimen motion relative to novel fixtures was assessed using a digitizer; fixture stiffness exceeded specimen stiffness by an order of magnitude. Intradiscal pressure (IDP), measured using a fiberoptic pressure transducer, confirmed rigidity and compressive force selection. Surrounding media was controlled at 37 °C, 5% O₂/CO₂ using a closed flow loop with an hypoxic incubator and was validated with probes in the specimen chamber. FSUs were subjected to cyclic compression (20 cycles) and four-hour creep at 1.0 MPa. Disc tissue was analyzed for cell viability using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which showed high viability (> 90%) regardless of loading. Conditioned media was assayed for type-II collagen degradation fragments (CTX-II) and an aggrecan epitope (CS-846) associated with new aggrecan synthesis. CTX-II concentrations were not associated with loading, but CS-846 concentrations appeared to be increased with loading. Preservation of the full FSU allows physiologic load transmission and future multi-axis motion and identification of load-responsive proteins, thereby forming a new niche in intervertebral disc organ culture.

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Conflict of Interest Statement

The authors have no financial conflicts of interest relating to this device development or research study.

Keywords

Mechanobiology; functional spinal unit; organ culture; intervertebral disc; axial compression

Introduction

Intervertebral disc (IVD) degeneration is a leading cause of low back pain, a prevalent debilitating condition that amounts to an annual economic burden approaching \$100B (Dagenais et al., 2008). Mechanical loading of the IVD, as in comparable degenerative conditions like osteoarthritis, can initiate and accelerate degeneration (Kroeber et al., 2002; Lotz et al., 1998) or exert protective or reparative effects against degeneration (MacLean et al., 2003). Using ex-vivo systems, disc researchers have begun to identify detrimental and beneficial effects of loading on matrix homeostasis as a function of mode, magnitude, frequency, and duration (Setton and Chen, 2006). Generally, ex-vivo organ culture systems apply static and dynamic uniaxial compression to IVDs within an incubator and analyze cell viability, transcriptional changes, and protein changes (Gantenbein et al., 2006; Junger et al., 2009; Korecki et al., 2007, 2008; Lee et al., 2006; Wang et al., 2007). In IVD culture, adjacent vertebrae are removed and endplates are shaved to improve diffusion through the avascular tissue. However, resection prevents in-situ loading because posterior structures and endplates influence load transmission to the disc (MacLean et al., 2007; Shirazi-Adl and Drouin, 1987; van der Veen et al., 2008). Additionally, few researchers have assayed organ culture conditioned media for released matrix fragments or enzymes, indicators of biological response to loading. To explore these important issues, a system was designed to allow in-situ loading of the IVD through a novel ex-vivo mechanobiological system that preserves functional spinal units (FSUs) in a controlled environment and facilitates biological analysis of tissue and media. The objective of this study was to develop and validate this new testing system.

Materials & Methods

System validation assessed the following design goals: (1) flexible-walled chamber construction, (2) rigid fixation of the FSU, (3) controlled temperature and oxygen concentrations, (4) maintained cell viability, and (5) detection of matrix fragments in conditioned media.

Specimen Preparation

Freshly harvested, 4–12 month-old New Zealand White rabbit (animal use approved by local IACUC) lumbar FSUs were cleaned of muscle and adipose tissue, and superior endplates of superior vertebrae and inferior endplates of inferior vertebrae endplates were removed. Posterior ligaments and facet capsules were preserved. FSUs were encapsulated in 2kDa molecular weight cut-off (MWCO) dialysis membranes (SpectraPor7, 2000 MWCO/24 mm diameter, Spectrum Laboratories, Inc., Ranch Dominguez, CA) containing ~2 ml culture media (Dulbecco's Modified Eagle Medium—10% fetal bovine serum, 1% penicillin/streptomycin, 100 µg/ml ascorbic acid, and 0.02 M dextrose).

Chamber Design

Encapsulated FSUs were axially aligned within sterile, stainless steel fixtures and attached via rubber-capped set screws tightened against the irregular vertebrae. While designed to be compatible with both an axial testing machine (ATM) and a serial linkage robot, in this study fixtures were attached to the ATM only. Two layers of latex and nitrile rubber, sealed against the fixtures with O-rings and pipe clamps, connected the fixtures to form a flexible

chamber and allow perfusion (1.25 ml/min) of 200 ml media from an adjacent incubator (37 °C, 5% O₂/CO₂) (see Figure 1).

Rigid Fixation

Axial fixture rigidity in cyclic (20 cycles) and constant (20 min) compression at 0.1 mm/s was assessed using a Faro Arm digitizer (Titanium Series Faro Arm, FARO Technologies, Inc., Lake Mary, FL) with a calibrated accuracy: ± 0.02 mm and point collection repeatability of <0.05 mm. FSU and fixture-vertebra stiffness were calculated and differences in stiffness were expected to be an order of magnitude apart for minimal contribution ($\sim 10\%$, set a priori) of fixation laxity to outcomes.

Intradiscal pressure (IDP) of the nucleus pulposus (NP) was measured in force-controlled axial compression using a 0.35 mm fiber-optic pressure probe (Samba Preclin 360 MR, Samba Sensor, Goteborg, Sweden) to relate applied force to target pressures. IDP measurements were made at the putative center of the NP using a mid-disc height, lateral approach. Forces were prescribed based on $IDP = F_a/A_c$ where A_c was calculated from Vernier calipers measurements, $A_c = (\pi/4)(L_{AP})(L_{LAT})$ (assumes elliptical geometry and uniform force distribution) (Beckstein et al., 2008). Both fixture rigidity and IDP were assessed in N=5 FSUs in cyclic preconditioning (10–15 cycles) and creep (constant force for 20 min) at force-target, F_a , aimed to achieve 1.0 MPa IDP, which is representative of mild physical activity (Wilke et al., 1999).

Environment

Chamber media temperature was monitored with an accurate (± 0.08 °C) temperature probe (P-M-1/10-1/8-6-O-T-3, OMEGA Engineering, Inc., Stamford, CN) via a pre-calibrated data acquisition device (PT-104, OMEGA). Temperatures were controlled at 37 °C (± 0.5 °C) with flexible heaters (1"x2" patches, 24V @ 0.8A, Electro Flex Heat, Inc., Bloomfield, CT) adhered to chambers and connected to a programmable voltage supply. Dissolved oxygen concentrations were controlled by material selection and measured with a dissolved oxygen probe (MIL-70, Microelectrodes, Inc., Bedford, NH).

Cell Viability

Cell viability in annulus fibrosus (AF) and NP was assessed using a colorimetric method based on reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at active mitochondria (Csonge et al., 2002). After 2 hour immersion in MTT (1 mg/ml), AF and NP separation, and 24 hour organic extraction using 2-Methoxyethanol (CHROMASOLV® for HPLC, Sigma-Aldrich, Inc., St. Louis, MO), absorbance values ($\lambda = 570$ nm) of 200 μ l of extract were normalized per tissue sample by wet weight and compared to fresh ($t=0$) tissue samples. Viability was assessed over 30 hours in incubator unloaded culture (N=3)— flask-cultured, floating FSUs within a standard incubator; 4 hour viability was chosen for testing and confirmed for loaded (N=2) and unloaded (N=2) FSUs.

Conditioned Media Analysis

Media within the dialysis membrane was assayed for matrix fragments, including a breakdown fragment of type-II collagen, c-telopeptide-II (CTX-II), and a putative marker of aggrecan synthesis, CS-846 (Rizkalla et al., 1992). Undiluted samples were analyzed using ELISA kits for CTX-II (Serum Pre-clinical CartiLaps® ELISA Kit, Immunodiagnostic Systems Inc., Fountain Hills, AZ) and CS-846 (CS-846 ELISA Kit, IBEX Pharmaceuticals Inc., Montreal, Canada) following the manufacturers' instructions.

Data Analysis

Mean stiffnesses with standard deviations (N=5 preconditioning, N=3 creep) were calculated; comparisons between FSU and fixture-vertebra were presented as a ratio of specimen to interface stiffness. Similarly, mean IDPs with standard deviations (N=5) were calculated, and a Wilcoxon signed-rank test was used to evaluate differences between groups. Due to small sample size, only mean and standard error of viability data (N=3 for flask culture; N=2 for chamber conditions) and matrix fragments (N=2) were calculated.

Results

Fixture stiffness was approximately ten times higher than FSU stiffness in cyclic preconditioning and constant compression (Table 1).

Average peak IDPs in cyclic preconditioning and IDP in three phases of creep were depicted in Figure 2. IDPs were near the 1.0 MPa target in preconditioning but decreased with time during constant compression: 0.96, 0.72, and 0.64 MPa over the three phases. Differences were not significant.

Initial prototypes included a single latex membrane between fixtures and nylon tubing couplings; use of double layers of latex and nitrile rubber along with polycarbonate couplings maintained 5% O₂ (+/- 1%) in the chamber. Viability exceeds or remains near the target of 70% in incubator culture (Figure 3a). Figure 3b illustrates the effect of loaded and unloaded chamber culture relative to incubator culture and fresh discs. No apparent changes are noted in response to loading, and all conditions appear viable relative to fresh discs. From Figure 4, it is apparent that matrix fragments were readily detectable; CTX-II concentrations appear to be unrelated to loading, while CS-846 concentrations increase in loaded samples.

Discussion

The novel design presented here simulates in-vivo conditions by placing a rigidly attached FSU in a monitored, closed loop flow of hypoxic, temperature-controlled media. Tissue may be analyzed readily (e.g. mRNA expression) and the surrounding media volume is minimized with an inner dialysis membrane for detection of released proteins. The system was successfully validated for axial rigidity, stable environmental conditions, maintained cell viability, and detection of released proteins.

Typical IVD organ culture systems resect vertebra and endplates (at least partially) and apply axial compression to disc explants (Gantenbein et al., 2006; Junger et al., 2009; Korecki et al., 2007; Lee et al., 2006). In contrast, the goal of this system to preserve the FSU; thus, novel attachment to vertebrae is necessary. Fixture stiffness was validated to ensure known mechanical inputs. While retention of vertebra and endplates may limit culture duration (<30 hours based on preliminary testing) through reduced diffusion, it facilitates in-situ load transmission (MacLean et al., 2007; Shirazi-Adl and Drouin, 1987; van der Veen et al., 2008), allowing study of acute response to complex loading. Flexible walls permit 6 DOF motion, and fixtures are compatible with a serial-linkage robot used for in-vitro flexibility testing of rabbit FSUs (Bell et al., 2009); future studies will explore FSU bending and torsion. Culture duration may be extended with pre-mortem heparinization to prevent capillary bud clogging (Lee et al., 2006).

The assumptions of disc geometry and load transmission presented in the methods for axial force prescription in an FSU warrant validation. Intradiscal pressure measurements confirm these assumptions, reinforce fixture rigidity assessment, and demonstrate expected time

dependent behavior. These temporal changes approximate patterns seen in-vivo (Botsford et al., 1994; Ekstrom et al., 2004).

Detection of large molecules in high concentrations in conditioned media demonstrates the utility of this system in identifying potential biomarkers of acute response to loading. Korecki et al. examined GAG content in conditioned media in a loaded IVD organ culture and observed increased GAG loss to media in response to loading (Korecki et al., 2007). Junger et al. measured slight increases in tissue levels of CS-846 in response to loading after three weeks (Junger et al., 2009). Although formal statistical significance could not be established with the small samples used in this study, CS-846 media concentrations appear to be load responsive. This change is likely indicative of released GAG and not new aggrecan synthesis. The load-responsiveness of CS-846 but not CTX-II suggests acute changes involve proteoglycans but not collagen, which reflects previously observed patterns in disc catabolism (Lyons et al., 1981).

While this model improves simulation of physiologic loading and permits analysis of conditioned media, this system faces clear limitations. Inclusion of vertebra and intact end plates reduces diffusion in to and out of the disc, which may limit long-term viability. Conditioned media proteins are not specific to the disc but include contributions from bone, ligaments, and facets. The choice of a rabbit model limits translation of these results to humans because of differences in size and cell populations. Additionally, the animal age range spans skeletal maturity (Kydd et al., 2005). Differences in cellularity and load transmission through the EP and NP may exist; however, rabbit NPs retain notochordal cells throughout adulthood (Scott et al., 1980), suggesting differences in cell population are less problematic than would be expected in other animal models (Miyazaki et al., 2009).

Future studies will explore transcriptional and protein changes in FSU tissue along with screening tests that may reveal novel load-responsive proteins that would serve as candidate biomarkers for in-vivo studies. This system may play a role in the translational research paradigm by bridging a gap between in-vitro and in-vivo studies of disc mechanobiology in simulating and evaluating complex, physiologic loading.

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References

- Beckstein JC, Sen S, Schaer TP, Vresilovic EJ, Elliott DM. Comparison of animal discs used in disc research to human lumbar disc: axial compression mechanics and glycosaminoglycan content. *Spine (Phila Pa 1976)*. 2008; 33:E166–E173. [PubMed: 18344845]
- Bell, KM.; Quan, BC.; Hartman, R.; Sowa, G.; Kang, J. Flexibility Testing for Evaluation of a Needle Puncture Rabbit Model of Disc Degeneration; 55th Annual Meeting of the Orthopaedic Research Society (ORS); Las Vegas, NV: 2009 March 2–5.
- Botsford DJ, Esses SI, Ogilvie-Harris DJ. In vivo diurnal variation in intervertebral disc volume and morphology. *Spine (Phila Pa 1976)*. 1994; 19:935–940. [PubMed: 8009352]
- Csonge L, Bravo D, Newman-Gage H, Rigley T, Conrad EU, Bakay A, Strong DM, Pellet S. Banking of osteochondral allografts, Part II. Preservation of Chondrocyte Viability During Long-Term Storage. *Cell Tissue Bank*. 2002; 3:161–168. [PubMed: 15256877]

- Dagenais S, Caro J, Haldeman S. A systematic review of low back pain cost of illness studies in the United States and internationally. *Spine J.* 2008; 8:8–20. [PubMed: 18164449]
- Ekstrom L, Holm S, Holm AK, Hansson T. In vivo porcine intradiscal pressure as a function of external loading. *J Spinal Disord Tech.* 2004; 17:312–316. [PubMed: 15280761]
- Gantenbein B, Grunhagen T, Lee CR, van Donkelaar CC, Alini M, Ito K. An in vitro organ culturing system for intervertebral disc explants with vertebral endplates: a feasibility study with ovine caudal discs. *Spine (Phila Pa 1976).* 2006; 31:2665–2673. [PubMed: 17077734]
- Junger S, Gantenbein-Ritter B, Lezuo P, Alini M, Ferguson SJ, Ito K. Effect of limited nutrition on in situ intervertebral disc cells under simulated-physiological loading. *Spine (Phila Pa 1976).* 2009; 34:1264–1271. [PubMed: 19455001]
- Korecki CL, MacLean JJ, Iatridis JC. Characterization of an in vitro intervertebral disc organ culture system. *Eur Spine J.* 2007; 16:1029–1037. [PubMed: 17629763]
- Korecki CL, MacLean JJ, Iatridis JC. Dynamic compression effects on intervertebral disc mechanics and biology. *Spine (Phila Pa 1976).* 2008; 33:1403–1409. [PubMed: 18520935]
- Kroeber MW, Unglaub F, Wang H, Schmid C, Thomsen M, Nerlich A, Richter W. New in vivo animal model to create intervertebral disc degeneration and to investigate the effects of therapeutic strategies to stimulate disc regeneration. *Spine (Phila Pa 1976).* 2002; 27:2684–2690. [PubMed: 12461394]
- Kydd AS, Reno C, Sorbetti JM, Hart DA. Influence of a single systemic corticosteroid injection on mRNA levels for a subset of genes in connective tissues of the rabbit knee: a comparison of steroid types and effect of skeletal maturity. *J Rheumatol.* 2005; 32:307–319. [PubMed: 15693093]
- Lee CR, Iatridis JC, Poveda L, Alini M. In vitro organ culture of the bovine intervertebral disc: effects of vertebral endplate and potential for mechanobiology studies. *Spine (Phila Pa 1976).* 2006; 31:515–522. [PubMed: 16508544]
- Lotz JC, Colliou OK, Chin JR, Duncan NA, Liebenberg E. Compression-induced degeneration of the intervertebral disc: an in vivo mouse model and finite-element study. *Spine (Phila Pa 1976).* 1998; 23:2493–2506. [PubMed: 9854748]
- Lyons G, Eisenstein SM, Sweet MB. Biochemical changes in intervertebral disc degeneration. *Biochim Biophys Acta.* 1981; 673:443–453. [PubMed: 7225426]
- MacLean JJ, Lee CR, Grad S, Ito K, Alini M, Iatridis JC. Effects of immobilization and dynamic compression on intervertebral disc cell gene expression in vivo. *Spine (Phila Pa 1976).* 2003; 28:973–981. [PubMed: 12768134]
- MacLean JJ, Owen JP, Iatridis JC. Role of endplates in contributing to compression behaviors of motion segments and intervertebral discs. *J Biomech.* 2007; 40:55–63. [PubMed: 16427060]
- Miyazaki T, Kobayashi S, Takeno K, Meir A, Urban J, Baba H. A phenotypic comparison of proteoglycan production of intervertebral disc cells isolated from rats, rabbits, and bovine tails; which animal model is most suitable to study tissue engineering and biological repair of human disc disorders? *Tissue Eng Part A.* 2009; 15:3835–3846. [PubMed: 19681728]
- Rizkalla G, Reiner A, Bogoch E, Poole AR. Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. *J Clin Invest.* 1992; 90:2268–2277. [PubMed: 1281828]
- Scott NA, Harris PF, Bagnall KM. A morphological and histological study of the postnatal development of intervertebral discs in the lumbar spine of the rabbit. *J Anat.* 1980; 130:75–81. [PubMed: 6154031]
- Setton LA, Chen J. Mechanobiology of the intervertebral disc and relevance to disc degeneration. *J Bone Joint Surg Am.* 2006; 88 Suppl 2:52–57. [PubMed: 16595444]
- Shirazi-Adl A, Drouin G. Load-bearing role of facets in a lumbar segment under sagittal plane loadings. *J Biomech.* 1987; 20:601–613. [PubMed: 3611136]
- van der Veen AJ, Mullender MG, Kingma I, van Dieen JH, Smit TH. Contribution of vertebral [corrected] bodies, endplates, and intervertebral discs to the compression creep of spinal motion segments. *J Biomech.* 2008; 41:1260–1268. [PubMed: 18328489]
- Wang DL, Jiang SD, Dai LY. Biologic response of the intervertebral disc to static and dynamic compression in vitro. *Spine (Phila Pa 1976).* 2007; 32:2521–2528. [PubMed: 17978649]

Wilke HJ, Neef P, Caimi M, Hoogland T, Claes LE. New in vivo measurements of pressures in the intervertebral disc in daily life. *Spine*. 1999; 24:755–762. [PubMed: 10222525]

Figure 1. Illustration of closed-loop mechanobiological system. Media is pumped from a sterile, hypoxic incubator through the specimen chamber. Heat is applied directly to the chamber, and temperature (T) and dissolved oxygen (DO) levels are monitored continuously. The functional spinal unit is axially loaded under the control of a custom-made axial testing machine.

Figure 2.

Mean nucleus pulposus (NP) intradiscal pressures (target of 1.0 MPa) during cyclic preconditioning and three phases of 20 min creep (crp): (i) 0–1 min, (ii) 8–10 min, and (iii) 16–18 min. Error bars represent standard deviations.

Figure 3.

Cell viability of (A) flask-cultured (incubator unloaded) functional spinal units from 0–30 hours in annulus fibrosus (AF) and nucleus pulposus (NP) tissue (N=3 each) and (B) flask-cultured (incubator unloaded), unloaded (chamber unloaded), and loaded discs for 4 hours in the specimen chamber (N=2 each). Weight-normalized absorbance (570 nm) is compared to discs isolated at time of harvest (t=0) (set to 1). Negative controls were desiccated for 48 hr prior to assay. Error bars represent standard deviations.

Figure 4. Chondroitin sulfate-846 (CS-846) and c-telopeptide-II (CTX-II) concentrations in conditioned media of inner membrane surrounding unloaded and loaded (4 hour) functional spinal units (N=2 each) within the specimen chamber. Error bars represent standard error of the mean.

Table 1

Mean axial stiffness (+/- standard deviation) (N/mm) of (i) rabbit functional spinal units (FSUs) in 10–15 cycles of axial compression (N=5) and 20 min creep (N=3) under 1.0 MPa load-control (0.1 mm/s ramping), (ii) the fixture-FSU interface, and (iii) the ratio of FSU-to-interface.

Axial Stiffness (N/mm)			
Motion	Fixture-Vertebra	FSU	Ratio
Cyclic	917.2 (606.9)	113.9 (38.3)	0.12
Constant	877.4 (171.4)	82.7 (0.97)	0.09