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Development and Validation of a Bioreactor System for Dynamic Loading and Mechanical Characterization of Whole Human Intervertebral Discs in Organ Culture

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Abstract

Intervertebral disc (IVD) degeneration is a common cause of back pain, and attempts to develop therapies are frustrated by lack of model systems that mimic the human condition. Human IVD organ culture models can address this gap, yet current models are limited since vertebral endplates are removed to maintain cell viability, physiological loading is not applied, and mechanical behaviors are not measured. This study aimed to (i) establish a method for isolating human IVDs from autopsy with intact vertebral endplates, and (ii) develop and validate an organ culture loading system for human or bovine IVDs. Human IVDs with intact endplates were isolated from cadavers within 48 hours of death and cultured for up to 21 days. IVDs remained viable with ~80% cell viability in nucleus and annulus regions. A dynamic loading system was designed and built with the capacity to culture 9 bovine or 6 human IVDs simultaneously while applying simulated physiologic loads (maximum force: 4kN) and measuring IVD mechanical behaviors. The loading system accurately applied dynamic loading regimes (RMS error <2.5N and total harmonic distortion <2.45%), and precisely evaluated mechanical behavior of rubber and bovine IVDs. Bovine IVDs maintained their mechanical behavior and retained >85% viable cells throughout the 3 week culture period. This organ culture loading system can closely mimic physiological conditions and be used to investigate response of living human and bovine IVDs to mechanical and chemical challenges and to screen therapeutic repair techniques.

Keywords

intervertebral disc; organ culture; bioreactor loading system; bovine; human

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Introduction

Low back pain is the leading cause of disability worldwide with huge socioeconomic costs that total more than \$100 billion annually in the United States alone (Katz, 2006; Vos et al., 2012). Intervertebral disc (IVD) degeneration is considered one of the key factors related to the development of low back pain (Andersson, 1999; Samartzis et al., 2013). Multiple attempts to treat IVD degeneration, such as injectable biologics or implants, have shown promise in animal models but have limited clinical success. Lack of an appropriate model for human disc disease remains a major obstacle for developing more effective treatments. Therefore, the overall goal of this study is to develop and validate a system in which human and large animal IVDs can be obtained, placed into culture, and maintained under physiological conditions. Utilizing such a system to screen potential therapies for IVD degeneration would accelerate the translation of repair therapies from “bench to bedside”.

Organ culture is a technique whereby whole IVDs are removed from the adjoining vertebrae and cultured ‘*ex-vivo*’ for varying durations. IVD organ culture models retain the natural complexities (slow transport phenomenon, cell/matrix interactions) of the native microenvironment while allowing high control over the mechanical and chemical boundary conditions. The use of human IVDs provides a clinically relevant model that incorporates both the age and disease related changes that accumulate over a lifetime. Prior studies have demonstrated the feasibility of whole human IVD organ culture, however, such models require removal of the vertebral endplates in order to prevent cell death (Gawri et al., 2011). The removal of endplates limits the scope of therapies that can be evaluated as it prevents the application of load and assessment of mechanical function. Multiple large animal models have shown that it is possible to retain the vertebral endplates and still maintain disc viability (Gantenbein et al., 2006; Junger et al., 2009). Therefore we investigated the feasibility of isolating human IVDs with intact vertebral endplates from autopsy, using techniques modified from large animal organ culture systems.

The human spine normally experiences loads that are both large in magnitude and dynamic in nature during activities of daily life. The compressive forces that the IVD experiences have been estimated to be up to ~2500N during dynamic lifting (Marras et al., 2001) and intradiscal pressures approach 1MPa during normal daily activities such as climbing stairs (0.5-0.7MPa) or jogging (0.35-0.95 MPa) (Wilke et al., 2003). The forces that the IVD experiences are expected to be higher under more extreme conditions. These high loads that develop during different activities emphasize the high magnitude of forces that are required in order to simulate physiologic conditions. Dynamic loading is also a critical component of any physiologic model as it plays an important role (i) in stimulating biosynthesis (Chan et al., 2011; Korecki et al., 2008; MacLean et al., 2004) and (ii) promoting molecular transport within the IVD (Huang and Gu, 2008; Urban et al., 2004). Therefore, culture systems must be able to dynamically apply high forces in order to simulate ‘physiologic’ loading that arises during normal activities. Multiple bioreactor systems have been designed for whole IVD organ culture (Chan et al., 2013; Haglund et al., 2011; Junger et al., 2009; Paul et al., 2012) however none have demonstrated the ability to apply the large forces that are required to simulate physiologic stresses commonly experienced by human IVDs.

For many therapies, the primary measure of success is the restoration of IVD height and mechanical function. Mechanical behaviors are also known to be sensitive to different types of injuries (Iatridis et al., 2013). Therefore, the assessment of mechanical function can provide an important tool to evaluate and compare repair strategies. Many emerging therapies aim to repair the IVDs structure using tissue engineering or biomaterial strategies which can involve biodegradable materials. Such materials require ongoing measurements of mechanical performance throughout the repair process as they integrate/interact with the native tissue. It is often challenging, or impossible, to assess how these treatments affect the mechanical behaviors of IVDs over time in *in-vivo* systems, or other model systems that retain the in-situ environment. Of the bioreactor systems available, only one has assessed mechanical properties during culture (Paul et al., 2012) and none are able to simulate the loads typically experienced by human IVDs. Therefore the development of a loading system that can assess changes in the IVDs mechanical function is a research priority.

The objectives of this study were to (1) verify the feasibility of using whole human IVDs with intact vertebral endplates for organ culture experiments and (2) to develop and validate a dynamic loading system capable of loading human and large animal IVDs which can also assess the mechanical properties of the IVD throughout culture. The design goals for this bioreactor system were that the system must be able to: (i) maintain viable cultures, (ii) fit within an incubator, (iii) apply simulated physiologic loads on human or bovine IVDs (Table 1), and (iv) assess mechanical behaviors and IVD height throughout culture. The validation of the mechanical performance of the dynamic loading organ culture system was performed using bovine caudal IVDs since they are readily available and approach the size of human IVDs.

Methods

Organ Culture Set-Up & Culture Conditions

Human and bovine IVDs were subject to similar cleaning and culturing processes, although additional safety precautions were taken for human specimens. Whole IVD organs (human & bovine) were isolated with intact vertebral endplates using a histologic band saw (Exakt 310, Exakt, Norderstedt, Germany), as previously described (Gantenbein et al., 2006; Illien-Junger et al., 2013). Following isolation, endplates were cleaned with a wound debridement system (Pulsavac®, Zimmer, Warsaw, IN) to remove potential blood clots. IVDs were then **briefly rinsed (~10-15 seconds)** with 70% ethanol and soaked in washing solution (3% penicillin/streptomycin and 1.5% fungizone in PBS) for 10 minutes. IVDs were then placed into culture at 37°C and 5% CO₂. Culture media consisted of high glucose Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 50µg/mL ascorbic acid, 1% penicillin/streptomycin, 0.5% fungizone (Fisher-Scientific, Waltham MA), and 1:500 primocin (Invivogen, San Diego, CA). All reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise noted.

Human Organ Culture—Permission was obtained for use of cadaveric material prior to specimen procurement. All spinal segments were graded via a previously established radiographic scoring system (Anterior-Posterior x-rays; Figure 1A) which correlates with

the Thompson morphologic grading scheme (Benneker et al., 2005; Thompson et al., 1990; Wilke et al., 2006). Highly degenerated IVDs (Thompson grade 5) were excluded from culturing, as these are not relevant to biologic treatments. To ensure that the spinal segment obtained from autopsy services was viable prior to the organ culture experiment, an excess piece of IVD tissue was checked for cell viability. Excess tissue consisted of either an adjacent IVD not used for organ culture or partial IVD tissue remaining at the superior or inferior end of the spinal segment. IVD tissue was placed into media containing thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) at 1mg/mL and incubated during the extraction process (Walter et al., 2011). If the tissue was not visibly blue (viable) by the end of the extraction process the cultures were not continued.

Four human lumbar IVD organs were isolated from three human spinal segments within 48hr after death (Table 2). Isolated IVDs were placed into a custom human organ culture chamber and cultured for 7, 14 or 21 days (Table 2) under either (i) diurnal loading (0.1MPa/0.2MPa) with weights changed every 12 hours or (ii) static loading (0.2MPa). The human organ culture chambers were designed and built to be identical in function to previously described bovine caudal IVD chambers (Lee et al., 2006; Oshima et al., 1993) but large enough to accommodate human lumbar IVDs up to 88mm in diameter (Figure 1B). The lower chamber has an inner diameter of 94mm, height of 70mm, and were fabricated from polyetherimide (Ultem®, Advanced Industrial, Cornelius, NC) which is autoclavable, easy to machine, and translucent for viewing media levels. Ultem® has a compressive modulus (ASTM D695) of ~3.3GPa which enabled mechanical loading with minimal deflections. Chambers also contain porous metal platens (Mott Corporation, Farmington, CT) that served as a permeable interface between the chamber and the vertebral endplates of the IVD organ culture. **The culture medium** was changed every three to four days.

Bovine Organ Culture (System Validation)—Twelve bovine caudal IVDs were harvested from six bovine tails obtained from a local abattoir (Green Village Packing Co., Green Village, NJ). Bovine culture chambers were newly fabricated from Ultem® and are nearly identical to those previously described (Lee et al., 2006; Oshima et al., 1993) with an inner diameter of 47mm and height of 90mm. IVDs were cultured for either 6 (N=6) or 20 days (N=6) under simulated physiologic loading modified from a previous study (Junger et al., 2009). Loading consisted of diurnal loading (8hr: 0.2MPa/16hrs: 0.6MPa) with 2 five-hour bouts of ‘exercise’ (0.6 ± 0.2 MPa @ 0.1Hz) during the daytime cycle. All cultures were held at 0.2MPa static compression until the start of the simulated physiologic loading. In order to have uniform durations of dynamic loading, the simulated physiologic loading began at 9AM on the day following organ culture set up which involved slight differences in timing of dissection, isolation, and preparation of each IVD. **The culture medium** was changed on days 4, 6, 10, 14, and 18.

Viability

Tissue viability was assessed on both human and bovine IVDs as previously described (Walter et al., 2011). Briefly, tissue was double stained with MTT to stain viable cells, and 4 ,6-diamidino-2-phenylindole (DAPI, Roche Diagnostics, Germany) to stain cell nuclei. Three 10 m thick sections were taken from each section and photographed at 10x or 20x.

Percent viability (Dual stained cells (MTT+DAPI) = live; DAPI only stained cells=dead) was calculated for each image and for both regions, annulus fibrosus (AF) and the nucleus pulposus (NP).

Bioreactor System Design

A hydraulically actuated bioreactor loading system, capable of fitting within an incubator, was built to physiologically load and mechanically evaluate 9 bovine IVDs or 6 human IVDs simultaneously (Figure 2). The system is capable of applying up to 4kN at 1 Hz and consists of a hydraulic pump (PP10/G2-005/3, Rexroth Bosch Group, Charlotte, NC) which is connected to three proportional hydraulic valves (4WREEM, Rexroth Bosch Group). Each hydraulic valve is connected to three hydraulic actuators (CHE 32-18-9A, Parker, Fairfield, NJ). Each actuator is instrumented with a 1000lb load-cell (06-1432-07 Honeywell, Columbus, OH) and a displacement transducer (LVDT, Omega LD620-10, Stamford CT) (Figure 2A). The pressure between the hydraulic valve and the hydraulic actuators is monitored via a pressure transducer (A-10, WIKA, Lawrenceville, GA). All electronics interface with a computer through a data acquisition system (USB-6259, National Instruments, Austin, TX) and are controlled using custom Labview software. The applied force was controlled via a proportional-integral-differential (PID) controller which minimizes the error between the averaged applied force and the force set point (Figure 2B).

Bioreactor System Validation

To validate the control system the root-mean-squared (RMS) error was calculated for the static and dynamic portions of the daily cycle. RMS error was calculated based on the difference between the set point and the control variable for one hydraulic valve over 12 independent trials. To additionally characterize the dynamic portion of the loading cycle the total harmonic distortion (THD) was calculated. The THD is the sum of the power of all the different frequency components of a signal relative to the fundamental frequency (0.1Hz) and is a measure of how closely the resulting signal resembles the set point. To assess whether mechanical parameters can accurately be determined, and to additionally validate the control system, the elastic modulus of a (25.4mm × 25.4mm × 12.7mm) piece of rubber was calculated on each piston and compared to that calculated when loaded by an Instron mechanical testing machine (DynaMight 8841, Norwood, MA). The loading protocol consisted of 100 cycles of 350±150N at 0.1Hz. The 100th cycle was used to determine the elastic modulus. The percent difference between the elastic modulus determined by the Instron and each individual piston was calculated.

Tissue Mechanics & IVD Dimensions

To validate that the dynamic loading system could monitor the mechanical function of multiple IVDs throughout the culture period multiple mechanical parameters were assessed each day. Mechanical parameters included the total amount of creep and recovery that occurred during each daytime and nighttime period, respectively, and the IVDs dynamic stiffness at the end of the 2nd dynamic loading period. The dynamic stiffness was calculated from the penultimate cycle of the 2nd “exercise” portion of the daily loading regime, after the IVD had undergone ~3599 cycles. These parameters were assessed as they directly measure the changes in IVD height and the functional ability of the IVD to bear load. IVD

heights were recorded at the set-up and takedown of experiments to assess changes in IVD height. All bovine height changes are relative to the 'equilibrium height', which is the IVD height at 9AM on Day 2 (after the IVD has undergone one complete diurnal cycle and fully recovered). The equilibrium height was used for normalization in order to account for swelling that occurred during the extraction process.

Statistics

To assess whether the loading system was able to maintain the IVDs mechanical function all mechanical parameters were compared at the beginning (Day 2) and end (Day 20) of the culture period using an un-paired t-test. All results are presented as average $\pm$ SD. All statistical analysis was conducted with GraphPad Prism 3 (La Jolla, Ca) with $p < 0.05$ considered significant.

Results

Bioreactor System Validation

The newly designed bioreactor system was capable of accurately applying the simulated physiologic loading (Figure 3). The RMS errors for the static and dynamic portions of the daily loading cycle were 0.8 ± 0.06 N and 2.5 ± 0.4 N respectively (Figure 3B-C). The total harmonic distortion, a measure of the strength of the harmonic frequencies relative to the forcing frequency (0.1Hz), was 2.45% (Figure 3D). The bioreactor system was also capable of accurately assessing the mechanical properties of rubber as compared to its properties on the calibrated Instron testing system (considered our gold standard). The elastic modulus of rubber calculated when loaded by the Instron was 8.556 MPa and the modulus calculated when loaded by each piston was within 3.04% (Figure 3E).

Tissue Mechanics & IVD Dimensions

The mechanical parameters of bovine IVDs were maintained throughout the culture period (Figure 4), which demonstrated (1) that the loading system can assess the IVDs mechanical function and (2) that the loading protocol did not alter the IVDs mechanical behavior. There were no significant differences ($p = 0.36$) between the dynamic stiffness at the beginning (Day 2: 1808.8 ± 264.8 N/mm) and end of the culture period (Day 19: 1936 ± 240.6 N/mm). There were also no differences in the amount of creep ($p = 0.65$) or recovery ($p = 0.62$) between the beginning (Day 2: Creep: $-8.6 \pm 1.8\%$ Recovery: $8.3 \pm 1.7\%$) and end of the culture period (Day 19: Creep: $-8.2 \pm 0.8\%$ Recovery: $7.8 \pm 0.9\%$). Minimal height loss was observed for bovine IVDs over the 20 day culture period with IVDs losing only $8.1 \pm 4.3\%$ of their height. Human IVDs maintained disc height under diurnal loading with a change of $-3.0 \pm 5.5\%$. However, human IVDs lost disc height when cultured under static loading with a change of -15.4% .

Viability

For human organ culture, all human IVDs remained viable for the entire culture period (Figure 5) demonstrating that human autopsy is a viable option for organ culture isolation. Human IVDs remained viable at both 14 (AF: $87.8 \pm 5.5\%$ & NP: $89.6 \pm 12.5\%$) and 21 days (AF: $80.7 \pm 10.8\%$ and NP: $75.4 \pm 13.4\%$), and some differences in viability may have been

associated with age and health at the time of autopsy. The observation that IVD tissue remained viable at 21 days also demonstrated that the retention of vertebral endplates did not impede nutrient transport. Bovine IVDs remained viable at 20 days with $92.5 \pm 7.7\%$ and $85.2 \pm 22.8\%$ viability in the AF and NP regions respectively.

Discussion

This study (1) verified the feasibility of isolating whole human IVDs from autopsy with intact endplates for use in organ culture and (2) developed, characterized, and validated a dynamic loading system capable of loading and mechanically evaluating both bovine and human IVDs. Human IVDs with intact endplates were obtained from autopsy within at least 48 hours post mortem and maintained viable in culture up to 21 days. The loading system that was developed can apply a wide range of loading conditions to either 6 human IVDs or 9 bovine IVDs simultaneously. The validation study demonstrated that simulated physiologic loading was able to maintain IVD height throughout the culture period and that the loading system can accurately assess the mechanical behavior of IVDs throughout culture period. This loading system gives us the capability to investigate questions related to intervertebral disc pathophysiology and repair on bovine or human IVDs.

Organ culture of whole human IVDs with intact endplates is an important methodological advance that allows a model that incorporates both the age and disease related changes that accumulate over a lifetime. It is under these conditions that screening and evaluation of clinically relevant repair strategies is most relevant. The application of human IVDs has many opportunities for advancing clinical translation (Gawri et al., 2013), but there are also many challenges with using human tissue. Specifically, the large variability between humans makes the identification of control levels an important issue. For example, a repeated measurement design with the selection of multiple IVDs from the same spine can reduce patient to patient variance, yet, there are commonly different levels of degeneration within the same human spine. Consequently, we may be limited to 2-3 IVD levels from a single cadaver limiting the numbers of interventions or time points. Alternately, Gawri et al. used control IVDs from different donors but matched for spinal level and degenerative grade (Gawri et al., 2013). Overall, human IVD organ culture may be best suited as a validation tool in the human system following prior screening and optimization tests in cell culture and small animal model studies.

Our viability results demonstrated that human IVDs, which retained vertebral endplates, were able to be maintained in organ culture for up to 21 days. This is in contrast to previous studies that found the retention of boney endplates resulted in cell death in all regions of the IVD within 7 days (Gawri et al., 2011). Our ability to retain high cell viability throughout culture is likely resultant to the thorough endplate cleaning that was previously shown to be critical in other large animal organ culture models (Gantenbein et al., 2006; Junger et al., 2009; Lee et al., 2006). The cell viabilities observed in our human IVDs are comparable to other large animal models that retain vertebral endplates, however, they are slightly lower than those reported in previous studies which cultured human IVDs with cartilaginous endplates and under free-swelling conditions (Gawri et al., 2011). We associated these differences with the retention of vertebral endplates; however, it is possible that these

differences are associated with viability measurement technique. We consider the advantages of retaining the vertebral endplate, such as more physiologic transport, the ability to apply loading and the capability to assess mechanical function, to outweigh any potential costs of slightly reduced viability.

The loading system developed in this manuscript is capable of accurately applying a wide range of loading regimes (with less than 3N of error) while being able to precisely monitor the IVDs mechanical response. Bovine IVDs largely retained disc height over the 20 day culture period, with only ~8% height loss, suggesting that applied loading was as appropriate baseline condition. The loading device was able to accurately determine the elastic modulus of rubber and this repeatability was also demonstrated through the multiple measurements taken throughout culture. There were no significant differences in any of the mechanical properties that were assessed throughout the culture period suggesting that the applied loading regime was not damaging. The dynamic stiffness' found in this study (1808.8 ± 264.8 N/mm) are slightly higher than those observed in a caprine organ culture model (1323.5 ± 251 N/mm) (Paul et al., 2013; Paul et al., 2012) and this is likely related to differences in species, although it may also be associated with differences in loading protocols or anatomical locations (lumbar vs. caudal).

Overall this study demonstrated (1) that it is possible to maintain viable IVDs organ culture, with intact vertebral endplates, for at least 21 days, and (2) describes the design, construction, and validation of an organ culture loading system that is capable of applying a wide range of loading regimes while also assessing the mechanical behavior of cultured IVDs. To our knowledge this is the first device that is capable of applying the high loads that the human IVDs typically experience. Human IVD organ culture together with the dynamic loading system designed in this study give us the capability to investigate the functional impact of biologic and mechanical repair (implants) strategies. This organ culture system is an important tool for the screening and validation of therapeutic repair techniques in a human model system.

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Figure 1. Human Organ Culture Procedure

A. IVDs within the spinal segments are graded via a radiographic scoring system to determine which IVDs are acceptable for organ culture. B. The culture system and culture chamber used for human IVD organ culture. C. A human IVD (L2/3) following 21 days of culture and cross-section.

Figure 2. Device Design & Control

A. Bioreactor loading system for bovine or human IVDs demonstrating three of the hydraulic pistons and organ culture chambers each instrumented with a load cell and linear variable differential transducer (LVDT – displacement sensor) B. A schematic representing the control and data acquisition (D.A.Q.) of the bioreactor loading system. Solid lines demonstrate flow of hydraulic fluid and dashed lines represent electrical signals.

Figure 3. System Validation

A. Simulated physiologic loading protocol. *Stress based on a 25.2mm Ø. RMS Error during the static (B.) and dynamic (C.) portions of the daily loading cycle D. Total harmonic distortion of dynamic loading portion E. Elastic modulus comparison of a piece of rubber as measured on an Instron mechanical testing device and each piston of the loading system.

Figure 4. Mechanical Organ Culture Validation

A. Applied load over one diurnal cycle (24hrs) B. The resulting displacement and the mechanical parameters that were assessed each day. There were no significant changes in the dynamic stiffness (C.), amount of creep that occurred during the day (D.) or the amount of recovery that occurred overnight (E.) over the culture duration.

Figure 5. Human Organ Culture Viability

A. MTT & DAPI viability images demonstrating viability within the Annulus Fibrosus (AF) and the Nucleus Pulposus (NP) of human IVDs after 14 and 21 days of culture. Scale bar = 100um B. Quantification of the viability in the different regions of the IVD.

Table 1

Loading System Force Requirements for Bovine and Human IVDs

	Average Force required for given Stress:				
	0.2 MPa	0.6 MPa	0.8 MPa	1 MPa	2 MPa
Bovine Caudal *	101 N	303 N	403 N	504 N	1008 N
Human Lumbar **	366 N	1097 N	1462 N	1828 N	3655 N

* Bovine Average Area (504 mm^2) N=23 IVDs

** Human Average Area (1828mm^2) N=14 IVDs

Table 2

Human Cadaver Information

Disc Level	Age	Sex	Culture Duration	Loading	Degenerative Grade	Change in Disc Height
Lumbar	8	F	14 Days	Static (0.2MPa)	1	-15.4%
T12/L1	53	M	7 Days	Diurnal (0.1/0.2 MPa)	3	3.2%
L2/3 L3/4	47	F	21 Days	Diurnal (0.1/0.2 MPa)	4 4	-7.3% (L2/3) -4.8% (L3/4)