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Dynamic Compression Effects on Intervertebral Disc Mechanics and Biology

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Abstract

Study Design—A bovine intervertebral disc organ culture model was used to study the effect of dynamic compression magnitude on mechanical behavior and measurement of biosynthesis rate, cell viability, and mRNA expression.

Objective—The objective of this study was to examine the effect of loading magnitude on intervertebral disc mechanics and biology in an organ culture model.

Summary of Background Data—The *in vivo* and cell culture response of intervertebral disc cells to dynamic mechanical loading provides evidence the disc responds in a magnitude dependant manner. However, the ability to link mechanical behavior of the disc with biologic phenomena has been limited. A large animal organ culture system facilitates measurements of tissue mechanics and biologic response parameters on the same sample allowing a broader understanding of disc mechanobiology.

Methods—Bovine caudal intervertebral discs were placed in organ culture for 6 days and assigned to a static control or 1 of 2 dynamic compression loading protocols (0.2–1 MPa or 0.2–2.5 MPa) at 1 Hz for 1 hour for 5 days. Disc structure was assessed with measurements of dynamic modulus, creep, height loss, water content, and proteoglycan loss to the culture medium. Cellular responses were assessed through changes in cell viability, metabolism, and qRT-PCR analyses.

Results—Increasing magnitudes of compression increased disc modulus and creep; however, all mechanical parameters recovered each day. In the annulus, significant increases in gene expression for collagen I and a trend of increasing sulfate incorporation were observed. In the nucleus, increasing gene expression for collagen I and MMP3 was observed between magnitudes and between static controls and the lowest magnitude of loading.

Conclusion—Results support the hypothesis that biologic remodeling precedes damage to the intervertebral disc structure, that compression is a healthy loading condition for the disc, and further support the link between applied loading and biologic remodeling.

Keywords

spine; intervertebral disc; organ culture; bovine; mechanics; dynamic compression loading

Experimental evidence points to a threshold of loading necessary for intervertebral disc (IVD) extracellular matrix maintenance, where too little load (*i.e.*, immobilization) will reduce biosynthesis rates and overloading can cause structural damage and altered biomechanical

behaviors.¹⁻³ Dynamic loading is commonly experienced during daily activity, and is particularly important to include when attempting to identify loading patterns that introduce risks to IVD structure, biomechanics, and biosynthesis. Furthermore, a cyclic loading component is necessary to distinguish between immobilization and overloading. *In vivo* studies demonstrated there is a frequency, magnitude, and duration effect of applied mechanical loading on IVD cells,^{2,4} further supporting the importance of a better understanding of such loading patterns on the IVD.

The motion segment complex provides 6 degree of freedom mobility, but its structural components are sensitive to damage under distinct loading conditions. Complex loading regimes (*e.g.*, bending and compression), on the spine can result in disc damage and herniation.^{3,5-8} Compression loading on the spine is known to put the vertebral endplate at risk of fracture, which is then associated with a loss of nucleus pressurization because of damage at the discovertebral junction.^{6,9} Evidence of biologic remodeling in disc tissue occurs in response to compressive loading magnitudes insufficient to cause vertebral endplate failure,² raising the possibility that thresholds of structural failure overestimate the levels of loading which are detrimental to intervertebral disc health.

The biologic response of the IVD to dynamic loading has been previously examined *in vivo*^{1,2,10-12} and in cell culture studies,^{4,13,14} whereas the effects of mechanical loading on IVD structure and mechanics have been studied extensively on nonviable tissue *in vitro*, leaving unanswered questions about the effects of such mechanical changes on living cell populations. Mechanical loading is known to influence the IVD; however, unanswered questions remain regarding the dependence on other signaling pathways existing *in vivo* (*e.g.*, proinflammatory molecules), and whether the loss of cell-tissue matrix contact *in vitro* is detrimental to normal mechanical signal transduction. The ability to examine biologic remodeling pathways while also quantifying structural and mechanical changes induced in response to mechanical loading is a critical step towards understanding how the relationship between biomechanical loading and biologic remodeling might contribute toward a progressive degenerative cascade in the IVD.

The use of an organ culture model facilitates investigation into cellular responses to mechanical loading while the disc is largely intact. Organ culture provides complete control over mechanical boundary conditions while allowing for measurement of mechanical properties throughout the culture duration. Chemical boundary conditions can also be controlled, eliminating the effect of other signaling pathways present *in vivo*, while maintaining viable cells and normal cell-matrix interactions. Currently, however, few studies have investigated the response of the IVD in organ culture to dynamic loading. Developing and testing a large animal organ culture system is important because of its ability to be more directly translated to human IVDs and also because of the ability to evaluate multiple mechanical and biologic dependent variables on the same IVD.

The aim of this study was to examine the effects of varying physiologic magnitudes of dynamic compression on intact intervertebral disc structure, biomechanics, cell metabolism, and water content in 3 disc regions. The hypotheses were that low magnitudes of dynamic compression would enhance anabolic remodeling whereas high magnitudes of dynamic compression would demonstrate early signs of disc damage and catabolic remodeling. Specifically, dynamic compression applied to the intervertebral disc structure at low magnitudes of active physiologic loading in a human (0.2–1 MPa, *e.g.*, standing up from a chair¹⁵) will promote anabolic remodeling, including increased biosynthesis rates, whereas loading at larger magnitudes of active physiologic loading in a human (0.2–2.5 MPa, *e.g.*, lifting 20 kg with round back¹⁵ but less than failure of bovine caudal motion segment^{16,17}) will result in early signs of remodeling including structural damage, loss of cell viability, and catabolic remodeling as measured

through biomechanical properties, histology, biochemical measurements, sulfate incorporation, and qRT-PCR.

Methods

Three intervertebral discs, corresponding to caudal levels C2–C3, C3–C4, and C4–C5, were dissected from 12 beef cattle (ages 18–24 months) under sterile conditions within 4 hours of slaughter. Dissection included removal of vertebral endplates from the intervertebral disc using a straight edge razor blade to maintain transport through the endplate route.¹⁸ After dissection, discs were rinsed in Tyrode's balanced salt solution (TBSS) containing 0.3 $\mu\text{L/mL}$ penicillin/streptomycin and 0.1 $\mu\text{L/mL}$ fungizone (Invitrogen, Carlsbad, CA).

Discs were assigned to 1 of 3 groups consisting of 2 dynamic loading conditions (low, high) and 1 static control (static). Each group consisted of $N = 12$ discs, with equal numbers of each disc level assigned to each group as the anatomic level of the disc is known to affect cell metabolism and tissue composition. Discs were placed into a custom built organ culture chamber described previously^{18,19} and housed in an incubator at 37°C and 5% CO_2 . Culture media consisting of DMEM (4.5 g/L glucose, 110 mg/L sodium pyruvate, with L-glutamine), supplemented with 100 U/mL of penicillin/streptomycin, 0.1 mg/mL gentamicin, 0.75 mg/L fungizone, 0.02 M HEPES buffer, 50 $\mu\text{g/mL}$ ascorbic acid (Invitrogen, Carlsbad, CA), and 10 mL/L FBS (Atlanta Biologic, Atlanta, GA) was continuously circulated at 1.1 mL/min and replaced every 2 days.

All groups were initially confined with an applied stress of 0.2 MPa for 12 hours.¹⁹ Chambers were then individually attached to an incubator-housed loading device for the start of the experimental protocol (Figure 1). First, a 1-minute test (0.2–0.4 MPa, 1Hz) was applied sinusoidally to obtain a preloading dynamic nominal modulus for all groups. Control discs (static group) were removed from the loading device and the 0.2 MPa static load was replaced, and a dynamic load was applied to the 2 test groups (low and high). Dynamic loading consisted of 1 hour of sinusoidal loading at 1 Hz, with amplitudes of 0.2 to 1 MPa for the low load group and 0.2 to 2.5 MPa for the high load group. After the dynamic loading cycle, a repeat of the 1-minute test was performed to obtain a postloading nominal dynamic modulus. After the 3 test cycles, the baseline 0.2 MPa static load was again applied to each chamber and at least 12 hours of recovery was allowed between dynamic load cycles. Each chamber experienced loading once per day, adding to 5 total times during the 6-day culture period.

Creep during 1 hour of dynamic loading was calculated from displacements that were recorded from the loading device at points corresponding to 0.2 MPa load for the first and last cycles of the 1 hour dynamic loading test, and the initial height at the first cycle was compared between days to compare height lost over the culture duration. Dynamic stiffnesses were calculated using custom written MATLAB code (The MathWorks, Natick, MA) and for ease of comparison across animals, all stiffness measurements were normalized by initial IVD cross-sectional area and presented as a “nominal modulus.”

Structural parameters assessed included changes in intervertebral disc diameter and height. Initial height and diameter measurements were obtained using 3 caliper measurements in each dimension, recorded before the start of the culturing process, and immediately after culture termination. Proteoglycan content released to the culture media was assessed using the DMMB colorimetric assay. Aliquots of culture media were centrifuged at 10,000 rpm for 3 minutes before the application of the DMMB assay. A standard curve was generated using chondroitin-6-sulfate and DMEM, and sample absorbances were read on a microplate reader. Regional tissue water content was calculated after tissue dissection, weighing, and lyophilization to obtain a dry tissue weight.

Cell metabolism was assessed using the ^{35}S incorporation assay. Immediately after culture termination, sections of intervertebral disc tissue from each of the tissue regions (OA, IA, NP) were dissected (Figure 1), weighed, and placed in 2 mL of culture medium without FBS containing 2.5 μCi of ^{35}S (Perkin-Elmer, Boston, MA) and brought to an approximate osmolarity of 400 mOsm by the addition of 1.5% v/v 5 M NaCl and 0.4 M KCl to reduce tissue swelling. Samples were incubated for 6 hours at 37°C and 5% CO_2 , after which they were removed from the radiolabel medium and digested with proteinase-K (0.5 mL of 1 mg/mL at 57°C). Radioactive media was stored for each tissue sample to allow for later normalization. After digestion, nonincorporated sulfate was removed by exhaustive dialysis against distilled water. Radioactivity of samples was measured using a scintillation counter, and was normalized to incubation media radioactivity and tissue sample dry weight. To minimize potential artifacts because of GAG leaching that may have occurred during the radiolabel incubation step, the sample dry weight was calculated based on measurements of specimen wet weight and water content of paired tissue samples that were taken before the incubation (Figure 1).

Cell viability was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO) to stain vital cells through the formation of precipitate by active mitochondria, and ethidium homodimer-1 (Invitrogen, Carlsbad, CA) to stain the DNA of nonvital cells with compromised nuclear envelopes. Tissue sections approximately 10 mm by 5 mm were dissected through the disc in the sagittal plane (Figure 1) and placed into a TBSS solution containing 1 mg/mL MTT and 1 $\mu\text{mol/L}$ ethidium homodimer-1. After a 2-hour staining period, samples were removed from the stain solution and placed on a shaker in TBSS for 10 minutes to remove excess dye. The tissue was then frozen in isopentane floated in liquid nitrogen and stored at -80°C until sectioning on a cryotome. Five 10 μm thick sections were taken for each tissue region (OA, IA, and NP) beginning at the tissue surface and every 250 μm thereafter. For each of the 15 resulting slides, representative images were captured at 20 $\times$ magnification (Zeiss axiocam, Zeiss, Thornwood, NY) first under fluorescent light to capture cells stained with ethidium homodimer-1 (Rhodamine filter: ex/em of 546 nm/617 nm) and then under brightfield light to capture precipitate formed with MTT by vital mitochondria.

QRT-PCR was performed on tissue isolated from the anulus and nucleus regions. The OA and IA were pooled to ensure sufficient tissue quantity for RNA isolation. Expression levels were quantified using SYBR green for aggrecan, versican, collagen types I and II, TIMP-1, MMP -2, -3 and -13, and ADAMTS-4 were normalized to 18S expression levels (to generate ΔCt values). Expression levels for experimental groups (low and high) were normalized to tail-matched static controls (to generate $\Delta\Delta\text{Ct}$ values).

A one-way ANOVA was used to evaluate the effect of loading group (static, low, high) on changes in disc mechanics, diameter, height, water content, GAG content in the media, and ^{35}S incorporation. All statistical analyses on qRT-PCR data were performed on the ΔCt values. A one way ANOVA was used to compare loading groups (low and high).⁸ Fisher PLSD *post hoc* test was used to detect differences between loading groups with a significance level of $P < 0.05$ for all ANOVA tests. For qRT-PCR data an additional Student *t* test with hypothesized mean of zero was used to evaluate statistical differences between static controls (static) and loading groups (low and high), which were normalized to the static controls.

Results

All data are presented as average $\pm$ SEM. No significant changes in disc height or diameter were found at the end of the culture period for any of the test groups. The average height loss was $14.8 \pm 1.95\%$ and diameter gain was $42.6 \pm 1.62\%$. Proteoglycan loss to the culture media was also not significantly affected by mechanical loading with an average of $0.065 \pm 0.004\%$

of the initial disc wet weight. Likewise, no significant differences in regional tissue water content were found between groups, with regional differences in tissue water content maintained. Average tissue water contents were $59.25 \pm 0.700\%$ in the OA, $73.59 \pm 0.617\%$ in the IA and $79.8 \pm 0.562\%$ in the NP. A trend of increasing sulfate incorporation (Figure 2) with increasing load magnitude was observed in the OA and IA; however, no corresponding trend was noted in the NP. Viability was not significantly different between loading groups (Figure 3).

The preloading dynamic modulus was not significantly different between loading groups at any time point (Figure 4). There was a significant increase in the preload modulus between day 1 and day 2 for all groups, but no further significant increases occurred throughout the culture duration for any group. The postload dynamic modulus significantly increased with increasing load magnitude. Likewise, the magnitude of creep observed during the 1 hour of dynamic loading was greater with increasing load magnitudes (Figure 5). No significant difference was noted in the starting disc height between days, indicating a complete recovery of the lost disc height between days.

Gene expression in the annulus region was not found to be significantly different with respect to static controls ($P > 0.05$) (Figure 6). Significant changes in collagen type 1 regulation were found between loading magnitudes, with downregulation observed in the low group and upregulation in the high group ($P = 0.48$). In the nucleus pulposus, significant upregulation of collagen type 1 ($P = 0.002$) and MMP3 ($P = 0.01$) gene expression was observed in the low group relative to static controls. Significant differences in gene expression were also observed between low and high loading groups for collagen 1 ($P = 0.018$) and MMP3 ($P = 0.01$).

Discussion

The effect of dynamic compression on the mechanical and biologic state of the intervertebral disc was examined at 2 loading magnitudes to test the hypotheses that low magnitudes of dynamic compression would increase anabolic remodeling whereas high magnitudes of dynamic compression would demonstrate early signs of disc damage and catabolic remodeling. The use of an organ culture system enabled measurement of mechanical properties throughout the culture duration while maintaining cell viability and metabolism, thus allowing for regional measurements of sulfate incorporation, qRT-PCR, histology, and water content. Overall, the results support the concept that the intervertebral disc tissue structure is tolerant to applied mechanical compression, with no observed permanent damage to the tissue structure. Load magnitude dependent increases in anabolic mRNA expression and sulfate incorporation suggest that dynamic compression increases disc metabolic rate and enhances anabolic remodeling. Significant differences in gene expression were also observed between loading magnitudes in both the annulus fibrosus and nucleus pulposus regions for collagen type I, and in the nucleus pulposus region for MMP3.

The magnitude of applied loading in this study was chosen to reflect expected magnitudes observed *in vivo*.¹⁵ The nominal dynamic modulus and creep magnitude of the disc increased with increasing load magnitude as would be expected due to nonlinear material behaviors associated with tissue compaction at higher loading magnitudes; however, all changes were fully recovered within 12 hours. The recovery times observed in this study are consistent with literature on the topic, with previous studies observing complete recovery after 18 hours.²⁰ Additionally, the removal of the vertebral endplates necessary to maintain cell viability¹⁸ has been shown to speed recovery time.²¹ Although the removal of endplates from the intervertebral disc is anticipated to affect absolute values of local strains in the intervertebral disc, it is not expected to affect relative comparisons between loading magnitudes. It is possible that the magnitude dependent increase in sulfate incorporation in the annulus fibrosus but not

the nucleus pulposus is associated with a loss of pressurization in the nucleus due to removal of endplates. The loss of disc height and increase in disc diameter observed is consistent with previous studies on the bovine intervertebral disc in culture,¹⁹ and is likely associated with postmortem muscle relaxation resulting in an increase in disc hydration.⁹ The full recovery of disc properties, combined with no significant changes in disc height, diameter, water content, and GAG loss to the culture media, provide evidence that applied compression loading results in no permanent damage to the intervertebral disc structure even up to magnitudes of 2.5 MPa.

Previous studies examining the biologic response of the intervertebral disc to dynamic loading have been performed *in vivo*^{1,2,12} and on cell cultures.^{4,13,14} Tissue culture and organ culture studies primarily examined the effects of static loading^{16,18,22} or diurnal loading (applied osmotically²³ or through compression¹⁹). The current study method links the response of isolated cells to that of the *in vivo* situation by retaining the *in situ* cell environment. It is possible that changes in the collagen microstructure may be present, but these did not affect the morphologic structural parameters that were measured in this study, and that are recorded in patients radiologically.²⁴ Although a direct comparison across studies and methodology is difficult due to the use of different species, and varying loading magnitudes, frequencies and modes of application (compression, hydrostatic, tension), it is fairly consistent that collagen type I and MMP3 are affected by applied mechanical stimulation in the intervertebral disc in a region specific manner.^{2,14}

In conclusion, increased cell metabolism as measured on the gene and protein levels was observed at loading magnitudes insufficient to cause observable disc damage, supporting the concept that biologic remodeling precedes intervertebral disc damage. Results also suggest that dynamic compression is a healthy loading condition due to the lack of observable signs of intervertebral disc damage at high stress levels in this study, combined with other studies on risky loading patterns on the disc that defined lateral bending and flexion as the loading patterns that place the disc at most risk of injury.⁸ Furthermore, it is interesting that load magnitude had a progressive increase in expression of many genes in the annulus while collagen-I and MMP3 in the nucleus region were all downregulated after a substantial increase in load magnitude. These observations demonstrate combined mechanical, biologic, and chemical remodeling in response to dynamic compression. Consequently, results motivate the need for further studies on the effects of applied loading on the biologic response of the intervertebral disc under more damaging conditions that might include bending and endplate fracture under compression.

Key Points

- The biologic and mechanical responses of the cultured intervertebral disc to applied dynamic compression were examined.
- No permanent compromise in disc mechanical properties was observed throughout the culture period.
- Biosynthesis rates and gene expression responses were affected by disc region and dynamic compression magnitude, particularly for collagen type 1 and MMP3.
- Dynamic compression magnitude increased biosynthesis rates and did not result in structural disruption, suggesting it is a healthy loading condition for the disc.
- Results suggest biologic changes occur before structural damage to the intervertebral disc.

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Dynamic Loading Protocol

Static Loading Protocol

Tissue Harvest

Figure 1.

Test protocol schematic detailing loading protocol (top). Illustration showing tissue harvest protocol (bottom). Note: Schematic diagram not to scale.

Figure 2.

Average $\pm$ SEM sulfate incorporation rates for all testing groups (OA, outer annulus; IA, inner annulus; NP, nucleus pulposus). A trend of increasing sulfate incorporation was seen in the annulus regions, but not in the nucleus.

Figure 3.

Representative viability images at 20 $\times$ magnification. Black = live, white = dead. Scale bar in black = 400 μ m. Columns represent tissue regions (OA, outer annulus; IA, inner annulus; NP, nucleus pulposus) and rows represent test groups. No changes in cell viability were observed.

Figure 4.
Average $\pm$ SEM nominal dynamic loading modulus for preload (top) and postload (bottom) tests.

Figure 5. Average $\pm$ SEM height loss for the 1 hour dynamic loading protocol. Significantly more height was lost at all time points in the high force dynamic loading groups than in the low force group.

Figure 6.

Gene expression fold change results as average $\pm$ SEM for anabolic (aggrecan, collagen types I and II, versican), anticatabolic (TIMP-1) (left column), and catabolic (MMP-2, -3, -13, and ADAMTS-4) (right column) genes for tissue from anulus fibrosus (top row) and nucleus pulposus (bottom row) regions. Significant differences ($P < 0.05$) between groups are marked with a * whereas significant differences from static controls (using a t test with hypothesized mean of zero) are marked with a § symbol (found in low groups in the NP for collagen type I and MMP3). Note that low and high groups are normalized to tail matched static controls.