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Free Axial Vibrations At 0 to 200 Hz Positively Affect Extracellular Matrix Messenger Ribonucleic Acid Expression in Bovine Nucleus Pulposi

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Study Design. Bovine caudal intervertebral discs (IVDs) were exposed to free axial vibration for 10 to 60 minutes at 0 to 0.5g and 0 to 200 Hz. Expression of messenger ribonucleic acid for aggrecan, collagen type I, collagen type II, biglycan, decorin, and versican were assayed, as was apoptosis.

Objective. To determine the vibration conditions which are most effective in altering intervertebral disc IVD gene expression.

Summary of Background Data. Various studies have suggested widely varying effects of vibration in the IVD, ranging from harmful (increased risk of degeneration) to beneficial (increased analgesia) to neutral (no effect).

Methods. Vibration was applied using a custom designed voice coil system, which generated controlled motion in the axial direction. Gene expression in the nucleus pulposus was assessed using RT-PCR and the SYBR green chemistry; apoptosis was assessed using TUNEL staining.

Results. Expression of messenger ribonucleic acids for biglycan, collagen type I, collagen type II, decorin, and versican were significantly affected by vibration duration, frequency, and amplitude. Aggrecan was unaffected. Of the 3 factors, amplitude had the largest and widest effect.

Conclusion. Expression of extracellular matrix genes was significantly upregulated at high amplitudes (>0.4 g) in as little as 10 minutes. This may indicate a potential therapeutic stimulus; periodic application of controlled vibration could positively influence matrix maintenance. Further studies on the protein level and long-term effects are warranted.

Key Words: intervertebral disc, gene expression, vibration, biomechanics, mechanical stimulation of tissues. **Spine 2010;35:1437–1444**

Chronic low back pain is a significant health problem in modern society. Low back pain is often associated with degeneration of the intervertebral discs (IVDs).[1] Numerous epidemiological studies have suggested various causes for disc-related back pain, including genetics,[2] smoking,[3] and loading history.[4] Of particular interest is the fact that an increased risk of disc-related back pain is associated with exposure to whole-body vibrations in the frequency range 2 to 11 Hz (ISO 2613).[5–7]

The IVDs provide mobility and a degree of shock absorbance to the spinal column. They also transmit forces between the adjacent vertebrae and prevent direct contact between the bones. It has been shown that the mechanical properties of the IVDs play an important role in their functionality.[1] Disc degeneration is often characterized by reduced disc height and stiffness, resulting in pressure on the radiating nerves. The dominant surgical treatment at present is spinal fusion, wherein 2 or more adjacent vertebral bodies are physically locked together using bone graft or instrumentation. Although this procedure often successfully restores disc height and allows for the return of a pain-free lifestyle, degeneration of adjacent motion segments (adjacent segment disease) is a common long-term complication. Although initially thought to be a rare event, adjacent segment disease is becoming more of a concern. One current theory states that by fusing dynamics of the joint, they are altered in a way that affects the healthy discs next to the fused segment, likely by altering loading and kinematics on these adjacent discs.[8–10]

The relationship between vibrations and low back pain has been studied since the 1950s. The main goal of those early studies was to mitigate low back pain often experienced by workers sitting and driving for long periods of time. However, the results of subsequent epidemiological studies have been somewhat mixed. When healthy (asymptomatic) individuals were compared with operators of heavy earth-moving machinery in an age-matched cohort, no differences were found with respect to water content, disc height, viscoelastic behavior, strength of the vertebrae as indicated by water content (magnetic resonance imaging) or bone density (QCT).[11] Similarly, when clinical and MRI assessment was performed on asymptomatic tractor driving farmers and a matched cohort, no difference was found in the degeneration of the spine.[12] In contrast, professional drivers have an increased risk of being hospitalized because of spinal disorders, with bus- and long-haul truck drivers having more frequent spinal disor-

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ders than other truck drivers, potentially because of their increased exposure.[13]

This study hypothesized that axial vibrations at physiologically relevant frequencies would have positive effects on expression of various extracellular matrix messenger ribonucleic acids (mRNAs). Bovine caudal IVDs were exposed to unconstrained axial vibrations at various frequencies for 10 or 60 minutes and probed for expression of aggrecan, biglycan, collagen type I, collagen type II, decorin, and versican mRNA.

■ Materials and Methods

Tissue Isolation

All procedures were approved by the University of Calgary's Animal Care Committee. Bovine tails were obtained from a local slaughterhouse within 6 hours of death. Muscle, fat, fascia, and the spinous processes were removed, and the IVDs were cut free using a bandsaw, leaving ~2 to 5 mm of bone on either side. The isolated IVDs were then stored in phosphate-buffered saline at 4°C until ready for experimentation.

All discs were visually inspected at the time of RNA harvest and found to be approximately equal to a human Thompson grade II disc (opaque fibrous nucleus, clear nuclear/anular demarcation, and distinct lamellae).

Vibration

Axial vibration was applied by placing individual discs into a chamber (Nalgene) filled with cell culture medium. The lid on the chamber was fixed with a spring (k = 26.2 N/cm) that applied static axial load (mean, 40.6 N) on the discs during the unconstrained vibration. The chamber had an ±1.7g accelerometer (ADXL 203, Analog Devices Inc., Norwood, MA) fixed to it to track the vibration load when the chamber was mounted to a voice coil (#NCM05-28-180-2LB, H2W Tech Inc., Vanencia, CA) (Figure 1). The accelerometer was previously calibrated using a "1g shaker" (Type 4291—1g Accelerometer Calibrator, Brüel & Kjaer, Copenhagen, Denmark). The vibration of the voice coil was controlled with the output of a Linear Current Amplifier Module (LCAM-1, Quanser, Markham, ON), which received its command signal from a function generator (PicoScope2203, Pico Technology, St Neots, Cambridgeshire). The LCAM was powered by 27 V, and cooled by a 7.06 CFM fan (#2412PS-12W-B30, NMB-MAT, China) to minimize temperature fluctuation of the output. The voice coil and chamber were secured with damping to a shelf in a 37°C and 5% CO_2 environmental control chamber. The control signal to the voice coil and the accelerometer output was monitored in real time through an oscilloscope (PicoScope2203, Pico Technology, St Neots, Cambridgeshire) during the loading. Vibration was applied at various frequencies (0, 8, 16, 20, 30, 40, 50, 60, 70, 80, 160, and 200 Hz) and amplitudes (0–0.54g RMS) for either 10 or 60 minutes. The order of both amplitude and frequency selection was randomly assigned to eliminate any time-dependent trends because of sample storage. All conditions were run on a minimum of 5 separate discs (from at least 2 different tails).

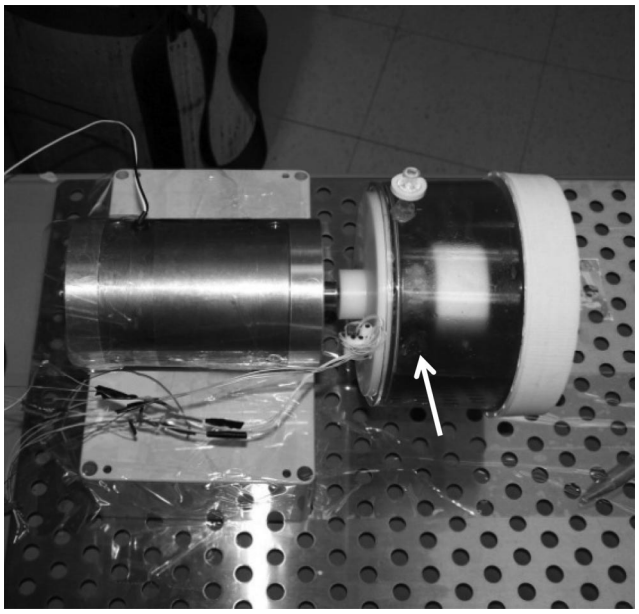

Figure 1. The vibration culture system. The IVD lies inside a polycarbonate culture chamber (right), immersed in DMEM culture medium (arrow). The culture chamber rests on the actuator end of a calibrated voice coil (left) and moves freely through the axial direction during vibration.

The treatment levels were determined from the results of a small pilot test (data not presented), wherein peak responses were found at 40 and 80 Hz, 60 minutes, and high-amplitude loads.

Control discs were treated equally (cleaned, stored, handled, dissected, and snap frozen) to perform as true unloaded controls. No analysis was performed at time 0 (before initiation of the vibration loading).

RT-PCR

At the end of the experimental period, discs were separated into nucleus pulposus and anulus fibrosus, flash frozen in liquid nitrogen, and stored at −80°C until extraction of total RNA. Only the NP was analyzed for this study; AF samples were stored for future testing, as pilot studies indicated minimal changes in the AF (data not shown). The frozen tissue was ground in Trizol reagent; full details of the protocol are provided elsewhere.[14] Briefly, total RNA was isolated using the Trispin method and quantified using the Ribogreen assay (Invitrogen). A sample containing 5 μg of RNA was reverse-transcribed using poly-T primers (First Strand Synthesis Kit, Stratagene). The resulting cDNA was probed with custom intron-spanning primers for aggrecan, biglycan, collagen type I, collagen type II, decorin, GAPDH, and versican (Table 1). Real-time PCR was performed using SYBR green chemistry (SYBR Green Premix, Bio-Rad) on an iCycler IQ system (Bio-Rad). Starting quantity was determined using the ddCt method, as calculated by the iCycler software. All data were normalized to GAPDH expression.

TUNEL

A subset of discs (1 per treatment for 0, 8, 16, and 80 Hz) were separated from the bone and fixed in 10% neutral buffered formalin. These discs were embedded in paraffin, sectioned at 8 μm, and mounted on glass slides. Random transverse sections throughout the disc were stained with either mercuric trichrome or TUNEL (Roche *In Situ* Cell Death Kit). TUNEL-positive and -negative cells were manually enumerated by 2 different observers on an upright microscope with a 40× objective.

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Table 1. PCR Primers and Thermocycler Settings

Gene	Forward Primer	Reverse Primer	Annealing Temperature
GAP	GGC GTG AAC CAC GAG AAG TAT AA	CCC TCC ACG ATG CCA AAG T	60
Aggrecan	GAG TGG AAC GAT GTC CCA TGT	GCA TTG ATC TCG TAT CGG TCC	50
Biglycan	GCT CCT CCA GGT GGT CTA TC	GCT GAT GCC GTT GTA GTA GG	50
Collagen I	AAG AAC CCA GCT CGC ACA TG	GGT TAG GGT CAA TCC AGT AGT AAC CA	50
Collagen II	GCA TTG CCT ACC TGG ACG AA	CGT TGG AGC CCT GGA TGA	50
Decorin	TGA CTT TAT GCT GGA AGA TGA G	TGG ACA ACT CGC AGA TGG	50
Versican	GAG AGT GTC GGT GCC TAC	GTC CTG TGT GTC TTC AAT CC	50

Data Analysis

Data were analyzed using the General Linear Model (GLM) to determine significant factors and interactions. The normality assumption was verified using normal probability plots and histograms of residuals. In those cases where substantial non-normalities were detected, Box-Cox analysis was performed and a suitable transform applied before reanalysis. Linear regression coefficients were determined to identify general trends in frequency or amplitude effects, and Tukey's *post hoc* test was used to compare individual treatments to control. Comparisons were considered significant at or below the $P = 0.05$ value.

One outlier data point was found (>2 standard deviations from the mean). However, removing it did not affect the results of the statistical analysis, so it was left in for the final analyses.

The figures presented here represent the results of the GLM analysis; therefore a plot for the effect of "Treatment X" represents the mean response across all other treatments. All bar graphs plot the mean ± standard error.

■ Results

The GLM analysis indicated that frequency significantly affected expression of collagen type II, decorin, and versican mRNA (Figure 2). The regression slopes for each of these genes were not significant. Amplitude significantly affected expression of biglycan, collagen type I, collagen type II, decorin, and versican mRNA (Figure 3). The regression slopes for these genes were significant and positive for all of these genes, with the exception of versican, which was not significant. Duration significantly affected expression of biglycan and versican, though neither regression slope was significant (Tables 2 and 3).

Because of the lack of a general trend in frequency response and the number of possible pairwise comparisons, only certain comparisons are presented here. Pairwise comparisons indicated that collagen type II was up-

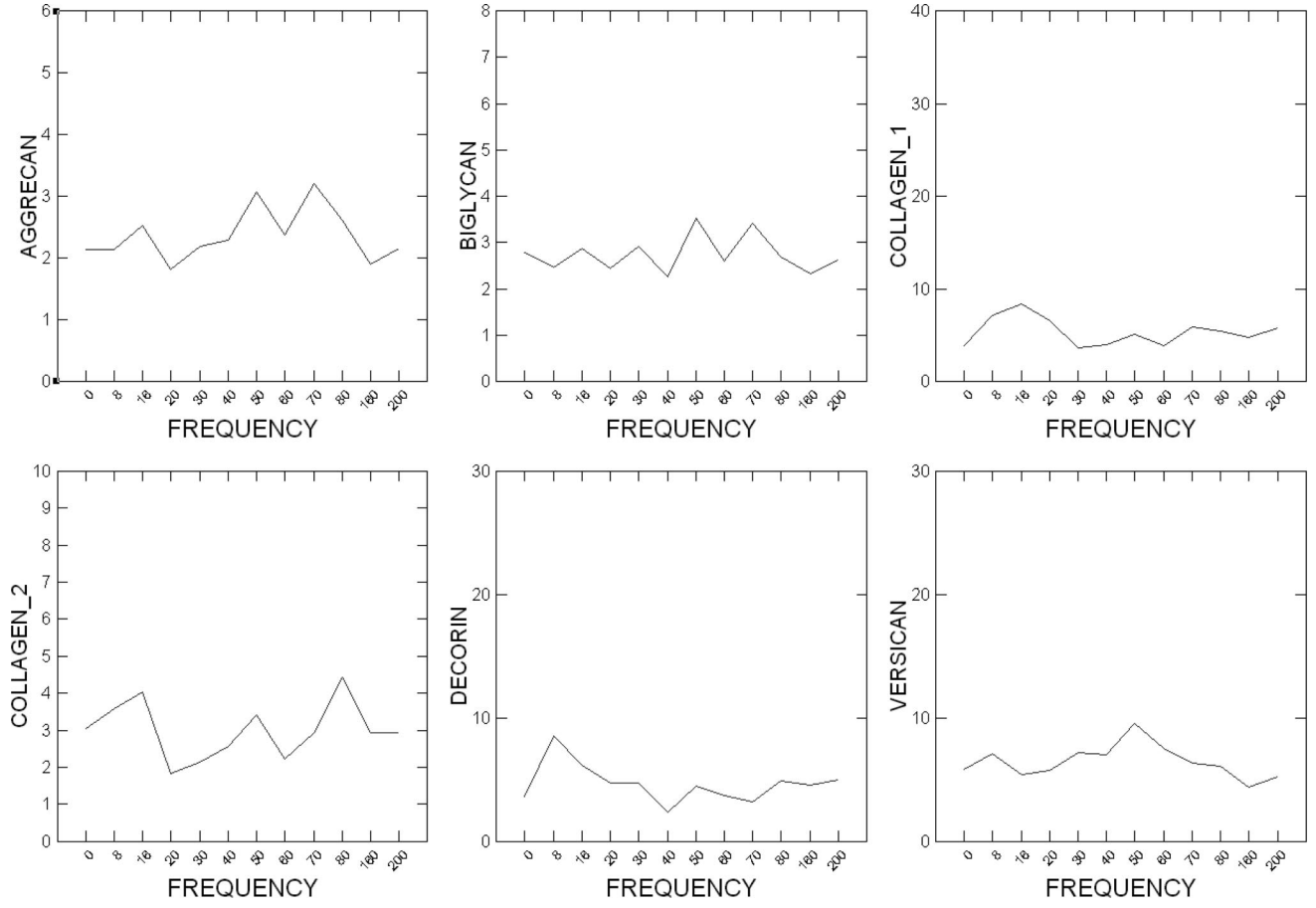

Figure 2. Gene expression trends with increasing frequency. No obvious qualitative trends were found.

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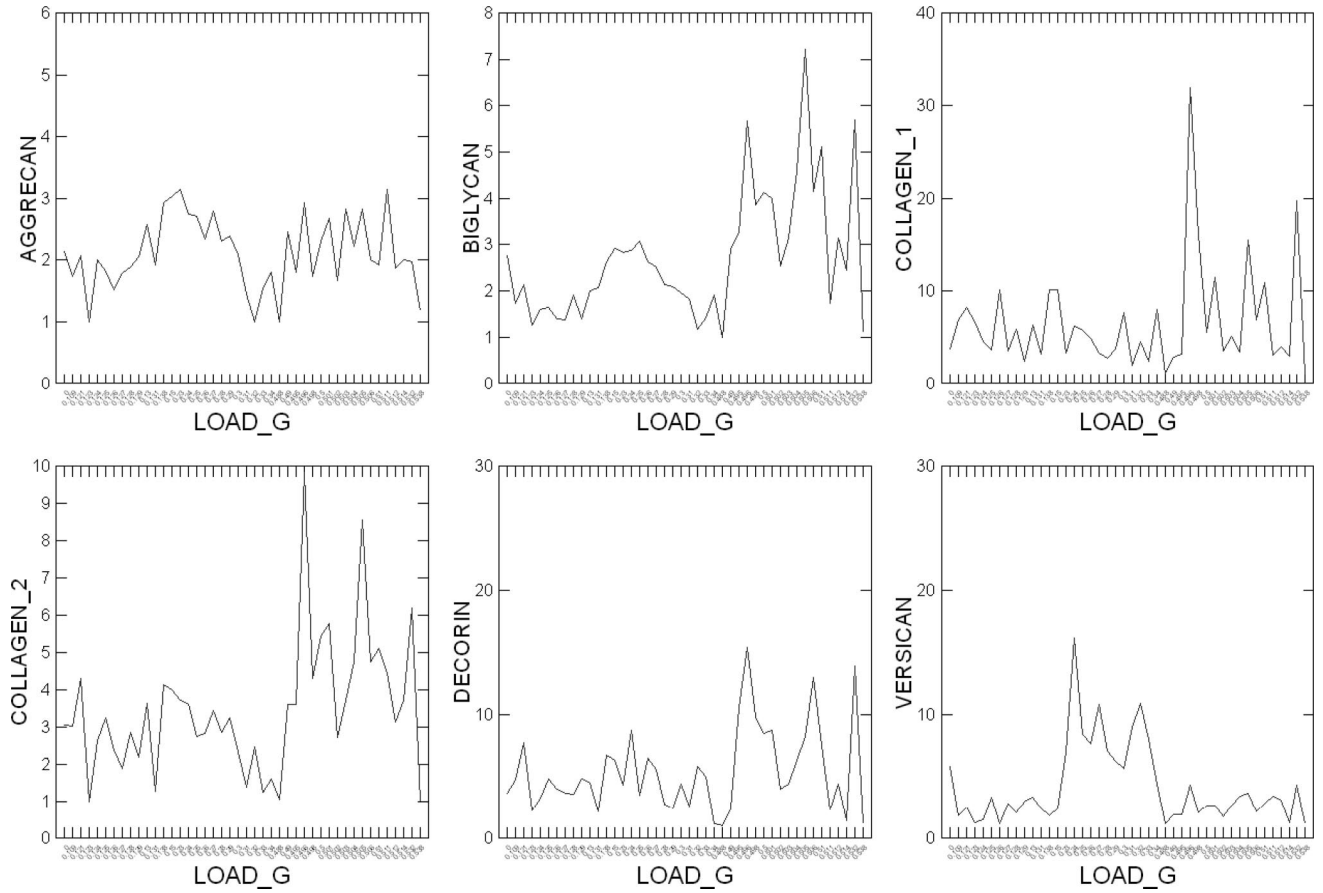

Figure 3. Gene expression trends with increasing load amplitude. Qualitative analysis suggested a threshold effect around 0.4 g; therefore, subsequent analysis sorted the treatments into control (no vibration), low amplitude (<0.4 g), and high amplitude (>0.4 g) conditions.

regulated at 80 Hz, whereas decorin was likewise upregulated at 8 Hz ($P = 0.027$ and 0.047, respectively) (Figure 4). In general, expression trends seemed to change around 0.49 g, so samples were lumped into general categories of "control," "low," and "high" amplitudes. Biglycan was significantly downregulated by vibration below 0.49 g and upregulated above 0.49 g ($P = 0.011$ and 0.020), whereas collagen type II and decorin were upregulated above 0.49 g ($P < 0.001$ and $P = 0.003$), and versican was downregulated above 0.49 g ($P < 0.001$) (Figure 5). No pairwise comparisons were significant for biglycan ($P > 0.17$ in all 3 comparisons). Versican was unchanged after 10 minutes of vibration but significantly downregulated after 60 minutes ($P = 0.41$ and $P = 0.040$) (Figure 6).

In histologic sections, the nucleus pulposus and anulus fibrosus were clearly defined and well formed, with no signs of degenerative changes, anular fissures, or other gross damage (not shown). TUNEL analysis indicated a mean background apoptosis rate of 10% ± 0.7% (mean ± standard error). There was no significant difference between frequencies ($P = 0.08$), amplitudes ($P = 0.44$), or anulus/nucleus ($P = 0.53$) (data not shown).

Table 2. *P* Values for Individual Factors and Genes From the GLM Analysis

	Aggrecan	Biglycan	Collagen Type I	Collagen Type II	Decorin	Versican
Frequency	0.139	0.417	0.237	0.033	0.004	0.007
Load (g)	0.914	<0.001	<0.001	0.010	0.026	<0.001
Duration	0.348	0.050	0.838	0.464	0.088	0.014

■ Discussion

Vibrations may have beneficial or adverse effects on musculoskeletal tissues. Various studies have suggested an increased risk of disc degeneration,[13] no effect,[12] or even an analgesic effect[15] of vibrations. Thus, further investigation of the cell- and tissue-scale processes is warranted.

Evaluation of mRNA changes is particularly challenging, because mRNA does not always directly correlate to functional changes in protein synthesis or matrix assembly. Some authors have chosen to ignore any changes below some arbitrary threshold; however, a clear justification for threshold choice is challenging. In this study, we chose to accept any statistically significant change as worth consideration, irrespective of potential "functional significance" of the change. Until further long-term tests on protein synthesis, matrix assembly, and

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Table 3. Regression Coefficients for the Significant Factors

	Aggrecan	Biglycan	Collagen Type I	Collagen Type II	Decorin	Versican
Frequency	—			ns ($P = 0.266$)	ns ($P = 0.057$)	ns ($P = 0.359$)
Load (g)	—	1.605 ($P = 0.001$)	4.209 ($P = 0.036$)	1.623 ($P = 0.025$)	8.383 ($P < 0.001$)	ns ($P = 0.622$)
Duration	—	ns ($P = 0.124$)				ns ($P = 0.206$)

ns indicates difference not statistically significant.

tissue quality can be performed, one must treat this— or any study on mRNA expression—with care.

In general, the results presented here indicate a positive effect of axial vibration on extracellular matrix gene expression. Most genes were at or above control levels for most frequencies and amplitudes, with the notable exceptions of biglycan and versican. Both of these genes exhibit complex expression patterns with high and low regions throughout the amplitude spectrum (Figure 3). Regardless of frequency and amplitude, versican expression was reduced after 60 minutes of exposure. It is possible that these proteoglycans are important in regulating the micromechanical environment during vibrations. Versican is a large proteoglycan with a molecular weight ~1000 kDa, with functions in cell adhesion and cell signaling. In contrast, biglycan is a small proteoglycan (~93 kDa) with functions in growth factor binding, and aggrecan is a large proteoglycan (>2800 kDa) with largely mechanical function in the tissue matrix.[16,17] It is difficult to speculate as to the micromechanical environment inside the tissue during vibration; however, it is possible that certain amplitude/frequency levels may induce unique stimuli. Further study, both experimental and theoretical, will be required to determine the underlying reasons for some of the trends observed here.

Most of the genes analyzed in this study are normally highly expressed in the nucleus pulposus; therefore, increased expression would be expected to correlate to tissue maintenance with vibration. However, collagen type I is normally expressed at a low level in healthy nucleus pulposi. Therefore, increased expression of collagen type I and decreased expression of versican may suggest a potential adverse affect of vibration. Further studies will be required to determine whether the positive effects (biglycan, collagen type II, and decorin) outweigh the negative effects (collagen type I and versican).

Some genes were found to vary abruptly at certain frequencies and amplitudes. This is not an artifact of the method, but rather a clear experimental finding. Only 1 data point was found to be a potential outlier (>2 standard deviations from the mean), and its removal had no effect on the statistical results. Furthermore, a repeatabil-

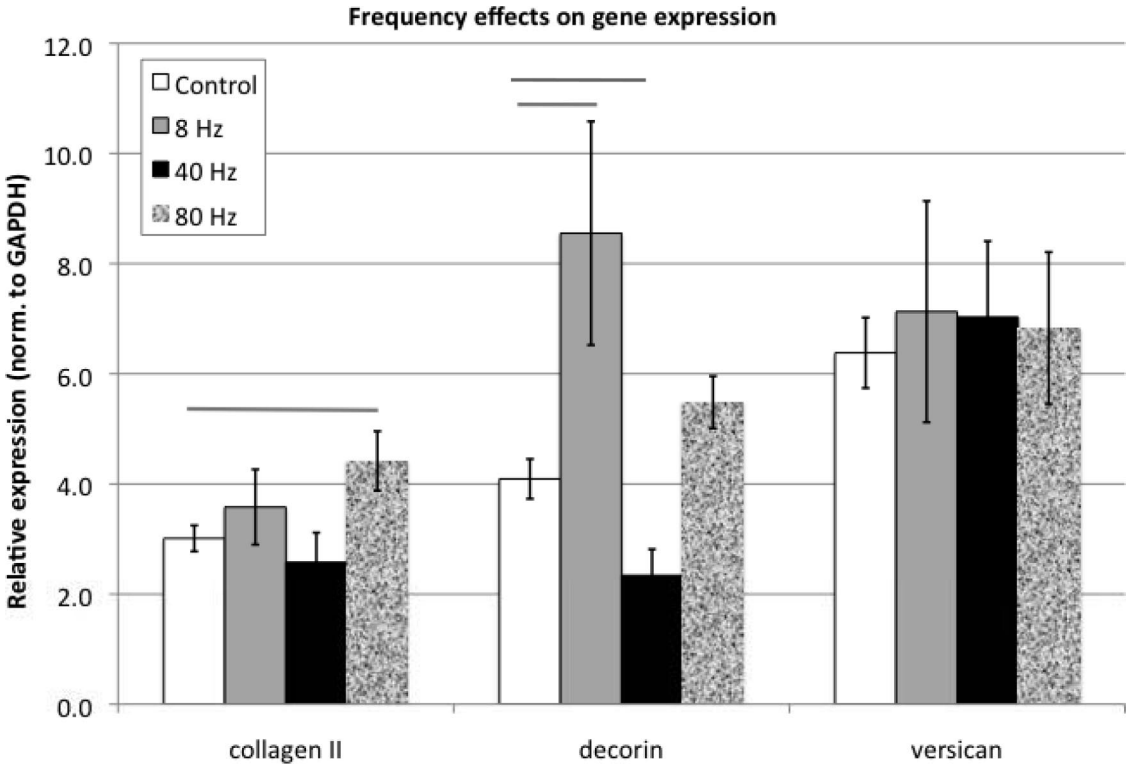

Figure 4. GLM analysis indicated a significant effect of vibration frequency on expression of collagen type II, decorin, and versican mRNA. Pairwise analysis indicated that collagen type II was significantly upregulated at 80 Hz, and decorin was significantly upregulated at 8 Hz (bars: $P < 0.05$).

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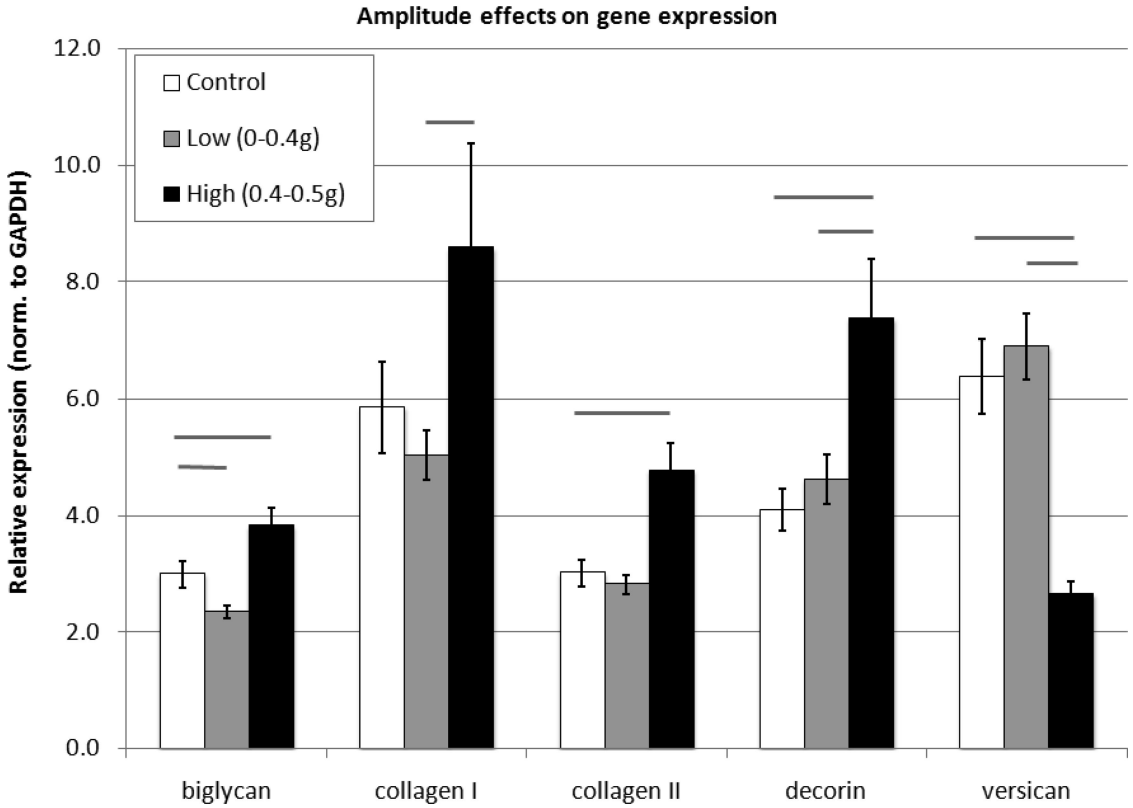

Figure 5. GLM analysis indicated a significant effect of vibration amplitude on expression of biglycan, collagen type I, collagen type II, decorin, and versican mRNA (bars: $P < 0.05$).

ity study of RT-PCR using the same techniques and chemistry as this study has found a variation in threshold cycle number (Ct) to be <1, which translates to ~5% error with each repeated sample run.[18] We took precautions to ensure that the same tissue preparation was used each time, and we randomized each loading condition to ensure that any time varying effect on samples would not hide trends in the data. Finally, a recently published study[19] has cited relatively larger standard errors than we have reported here for the same genes.

It is interesting to note that the small proteoglycans (biglycan, decorin, and versican) were influenced by vibration, whereas the large proteoglycan (aggrecan) was not affected. The current data are insufficient to determine whether the gene expression changes translate into altered protein expression. However, this difference may suggest a fundamental difference in the functionality of the large *versus* small proteoglycans during mechanical loading.

It is logical for genes of proteins of various sizes and functions to regulate expression responses differently as a result of the same loading condition. Recent published research shows that a 5-fold difference in gene expression (aggrecan and collagen II) can exist between genes under the same loading protocol. As for why this is occurring is difficult to say because much work is needed to delineate this question. However, mechanically, direct mechanotransduction leads to both extracellular matrix strain and hydrostatic pressure changes, which ultimately result in changes in cell shape and size. These changes in cell shape and size affect mechanoreceptors and stress-gated ion channels on the cell surface that in turn modify biosynthesis through multiple signaling pathways.[20] It is logical then that various sized cells responsible for producing specific proteins would undergo different relative strains for a given tissue displacement and thus have different cell signaling pathway responses leading to differences in mRNA signals. Include the fact that these same proteins have different functions and some researchers have stated that frequencies alone despite minimal tissue deformation can influence gene expression,[21] we can come to expect that expression between different genes will vary at the same loading condition.

It should be noted that this study applied unconstrained vibration. A 40-N tare load was applied to the disc, and the entire culture chamber was vibrated in the axial direction. This is distinct from other systems, which apply oscillating axial compression. Rapid motion of the chamber induces eddy currents in the culture medium and presumably increases nutrient transfer through the disc. A similar phenomenon would occur through convective pumping in the axial compression case, but the microenvironment will be different in the 2 cases.

It has been demonstrated that whole-body vibration causes altered expression of various neuropeptides in the dorsal root ganglions, adjacent to the IVD.[22,23] Recent evidence suggests that the degenerated IVD may contain nocioceptive fibers.[24,25] Taken together with the data

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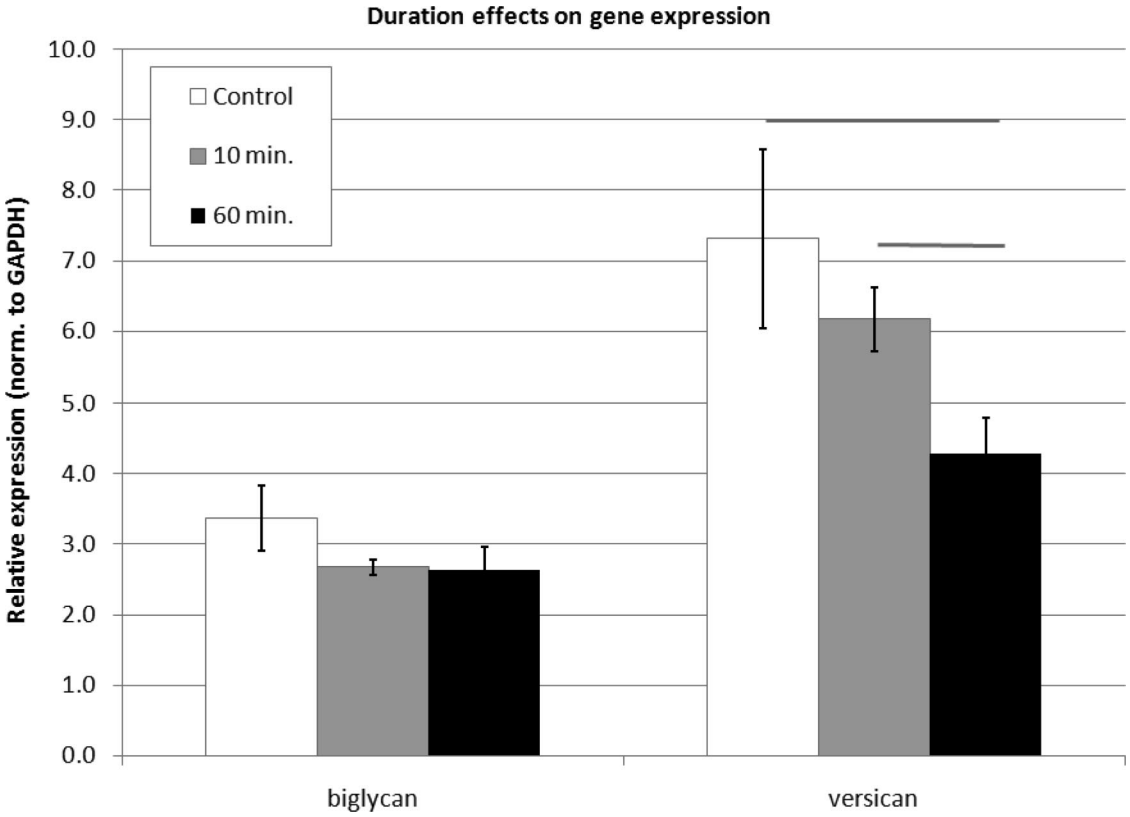

Figure 6. GLM analysis indicated a significant effect of vibration duration on expression of biglycan and versican mRNA; however, only versican demonstrated significant pairwise comparisons (bars: $P < 0.05$).

presented here, we begin to see a complex relationship between vibration, pain, and tissue maintenance. However, firm links cannot be drawn at this time, in part because of the different loading protocols used in the various studies, and further study is merited.

Taken as a whole, these results clearly indicate that vibration influences extracellular matrix gene expression. The manner of the influence (positive or negative) is yet to be determined, and further study is warranted.

■ Key Points

- Axial free vibration influenced expression of mRNA for biglycan, collagen type I, collagen type II, decorin, and versican in bovine nucleus pulposi.
- Aggrecan was unaffected by vibration.
- Vibration amplitude had the most substantial effect.
- Axial free vibration had no detectable effect on apoptosis rates.

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