

# Disc Strain and Resulting Positive mRNA Expression from Application of a Noninvasive Treatment

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**Study Design.** Bovine caudal intervertebral discs were exposed to a noninvasive vibrating intervention for 10 minutes at amplitudes of 0 or 0.5 to 5 g and frequencies of 0, 16, 50 to 80, and a combined 16 + 50 to 80 Hz treatment. Expression of mRNA for aggrecan, collagen type I, collagen type II, biglycan, decorin, and versican were assayed.

**Objective.** To determine if the intervention is effective in altering intervertebral disc gene expression.

**Summary of Background Data.** Studies have variously suggested either an increased risk of disc degeneration with vibrations, no effect, analgesic effect, or even positive effects within certain loading parameters. The KKT intervention is in clinical use for spinal ailment pain reduction.

**Methods.** The intervention was applied in a clinic emulation setup. Gene expression in the nucleus pulposus was assessed using real-time RT-PCR and SYBR Green chemistry.

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Spine

**Results.** Expression of mRNAs for aggrecan, collagen type II, and versican were significantly effected by the intervention. Collagen type I, biglycan, and decorin were unaffected.

**Conclusion.** Expression of the extracellular matrix genes were significantly up-regulated when vibrated with the intervention under specific loading patterns, indicating a potential therapeutic stimulus. Further studies on the protein-level and long-term effects are warranted. Previous studies have indicated a mixed effect of vibrations in the human spine. In this study, a clinical intervention using vibrations was applied to bovine intervertebral discs, and gene expression in the nucleus pulposus was measured. Several extracellular matrix genes were up-regulated, suggesting a potential therapeutic effect.

**Key words:** intervertebral disc, gene expression, vibration, biomechanics, mechanical stimulation of tissues, treatment. **Spine 2011;36:E921–E928**

Chronic back pain is a significant health problem associated with degeneration of the intervertebral discs (IVD).<sup>1</sup> Studies examining the association between back pain and degenerated IVDs with different approaches (e.g., examination of volunteers<sup>2</sup> and patients,<sup>3</sup> imaging investigations,<sup>4</sup> trials of intervention<sup>5</sup>) have produced strong evidence implicating the IVD as a significant factor in chronic back pain, leading to the use of the term “discogenic back pain.” Although the current justification of the clinical use of the intervention used in this study is spinal joint pivot point correction<sup>6</sup> and decreases in back pain,<sup>7</sup> the goal of this research is to show that it is possible for this intervention to cause upregulation of the genes responsible for maintaining disc matrix indicating potential for changes in tissue maintenance. Our long-term hypothesis is that this upregulation of the appropriate genes has a potential to offset disc degeneration altering pathogenesis of disc tissue. This may or may not alleviate the associated discogenic back pain but presents a mechanism that may cause long-term maintenance of pivot point correction.

Traditionally, treatment is varied and focused on the symptoms instead of at the root of discogenic back pain, the disc itself. The more conservative approaches include general exercise, specific conditioning of back and abdominal muscles to help stabilize hyper-mobile regions,<sup>8–10</sup> spinal manipulation to increase the range of motion for hypo-mobile regions,<sup>11–13</sup>

www.spinejournal.com E921

massage therapy,<sup>14–16</sup> and transcutaneous electrical nerve stimulation.<sup>17–19</sup> The more invasive treatments involve the use of medications such as analgesics, opiates, anticonvulsant agents, or antidepressants<sup>20–22</sup>; minimally invasive treatments such as acupuncture,<sup>14,16,23</sup> epidural, and facet joint corticosteroid injections,<sup>24</sup> and spinal nerve blocking techniques.<sup>25–27</sup> The most invasive treatments involve surgical intervention, ranging from microdiscectomy and spinal fusion to laminectomy.<sup>28–30</sup>

Despite the multitude of treatments and clinical studies, discogenic back pain still remains one of the most elusive ailments of our time and lacks standardized guidelines for treatment that uniformly achieve acceptable results.<sup>31</sup> In fact, within the framework of evidence-based medicine, the best treatment for discogenic back pain remains cognitive intervention combined with physical exercises specific for stabilizing the spine.<sup>9,12,30,32</sup> Therefore, there is a clear need for further high-quality studies of new approaches, focusing on the associated disc degeneration, to advance patient care.

The mechanical properties of the intervertebral discs play an important role in their functionality.<sup>1</sup> Disc degeneration is often characterized by reduced disc height and increased stiffness, leading to bulging or herniation that can create pressure on the radiating nerves and spinal cord. The dominant treatment at present is spinal fusion, wherein two or more adjacent vertebral bodies are physically locked together using bone graft or instrumentation. Although this procedure often successfully eliminates stenosis and restores disc height, thus reducing nerve pressure, degeneration of adjacent motion segments is a common long-term complication through negative changes in joint dynamics.<sup>33–35</sup>

Previous reports have studied the unconstrained axial vibrations at physiologically relevant frequencies and discovered positive effects on expression of various extracellular matrix mRNAs.<sup>36</sup> In this study, we hypothesized that a clinically approved device for applying transcutaneous vibrations to the spine (Khan Kinetic Treatment, KKT) may also have positive effects on gene expression, which has the potential of increasing the health of the disc, decreasing the associated ailments of disc degeneration. In the previous experiments, bovine tail muscle, fat, fascia, and spinous processes were removed, and the IVDs were excised, leaving approximately

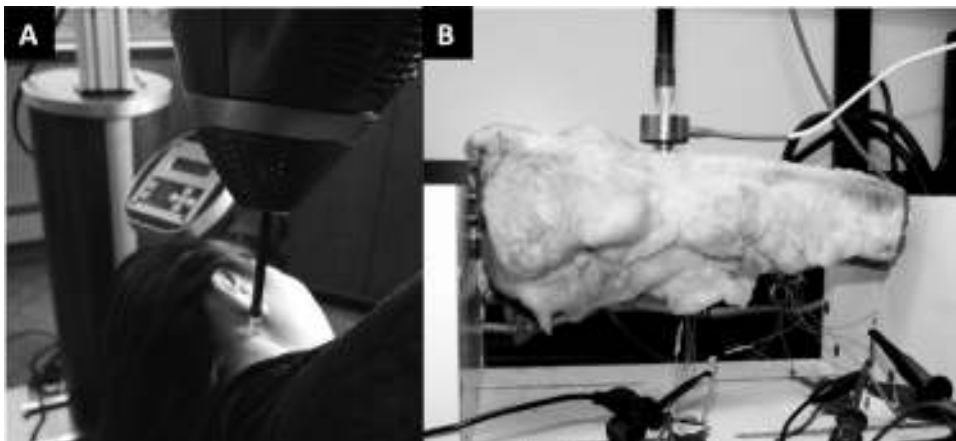
2–5 mm of bone on either side before loading. In this study, the bovine tail remained intact during loading to emulate clinical treatment with the KKT device (Figure 1). Limitations of this set-up include nonfunctioning muscle, animal anatomy as opposed to human, plus rigid rostral and caudal fixation as opposed to pseudo-rigid in the case of humans undergoing actual treatment. However, emulation is significant because muscle, adipose, and disc tissue act as low pass filters (damping ratios in the order of 0.19–0.23), so it is important to know the strain that actually reaches the disc level for a given input force and frequency with all tissues intact. Further, bovine discs have been considered a prime candidate for IVD mechanobiology studies because of their large size, similar aspect ratio, diffusion distance, and resting pressure (0.2–0.3 MPa) as compared to human discs. Bovine discs have also been found to be similar in composition, comparable hydration, collagen profiles, proteoglycan profiles, and similar rate of proteoglycan synthesis to human discs.<sup>37,38</sup>

Hence, intact bovine tails were exposed to vibrations using the KKT device for 10 minutes. We recorded the imparted motions at the disc level and probed for expression of aggrecan, biglycan, collagen type I, collagen type II, decorin, and versican mRNA.

## MATERIALS AND METHODS

### Tissue

All procedures were approved by the University of Calgary's Animal Care Committee. Bovine tails from skeletally mature cattle were obtained from a local slaughterhouse within 6 hours of death. Muscle, fat, fascia, and five vertebrae remained intact during loading, although the skin was removed by the abattoir. The two extreme caudal and rostral vertebrae out of the five-segment tail were scored with a bandsaw and fixed to a device that holds the tail segment in a way that emulated clinical positioning of the cervical spine (Figure 1). Figure 1(A) shows how the device is used in the clinic, head and shoulder fixed on treatment bench with neck freely suspended; similarly Figure 1(B) shows the clinical emulation test set-up using the bovine tail fixed at either end. The center vertebra was loaded with the treatment device.



**Figure 1.** A, KKT unit being used in the clinic on a patient. B, Five segment bovine tail clinical emulation set-up.

### Vibration Loading

Vibration was applied to the discs by placing the stylus of the KKT device onto the sensitive region of a 450 N load cell (Honeywell, Morristown, NJ) that was fixed over the area of the spinous process of the center vertebrae of the five-segment bovine tail (Figure 1B). Three-dimensional  $\pm 10$  g accelerometers (#MMA7261QT, Freescale Semiconductor, Inc., Austin, TX) were mounted on a cube and aligned with the axes of the disc (X-axial compression/tension, Y-shear 90° out of alignment with applied load, Z-shear parallel with applied load) and glued to the bone using cyanoacrylate to track acceleration of both the loaded and adjacent vertebral bodies. The accelerometers were previously calibrated using a 1 g shaker plate (Type 4291—1 g Accelerometer Calibrator, Brüel & Kjaer, Copenhagen, Denmark). The data from the load cell and accelerometer was collected via a PCMCIA data acquisition card (National Instruments, Austin, TX) at 3000 Hz using Labview 2009 and processed to produce three-dimensional disc strain using DIAdem 10.2 software packages (National Instruments). To obtain the specific vibration conditions of this project, KKTs internal control was bypassed. The voice coil mounted and producing the vibration from within the KKT unit 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, UK). The LCAM was powered by 27 V, and cooled by a 7.06 CFM fan (#2412PS-12W-B30, NMB-MAT, China) to eliminate temperature fluctuation of the output. The current going to the voice coil and the accelerometer output was monitored in real-time via an oscilloscope (PicoScope2203, Pico Technology) during the loading. Imparted mechanics vibration was tested at four different current values ( $\sim 0.9$ – $1.9$  Amp driving current). RT-PCR testing vibration was applied at two static frequencies (0 or 16 Hz) and/or one sweep frequency (50–80 Hz) that would step up the frequency by 2 Hz every two cycles of oscillation. Each frequency treatment was applied for 10 minutes and one treatment alternated combined frequencies of 16 and 50 to 80 Hz for 5 minutes each to maintain the overall 10 minute application. All amplitudes were sustained at 0.5 to 5 g peak root mean squared of the vertebrae directly receiving the load. This is similar to current clinical treatments using the device, and corresponds to those stimuli eliciting peak responses in previous experiments.<sup>36</sup> The order of control samples *versus* actual vibration samples was randomly assigned to eliminate any time-dependent trends because of sample storage. All conditions were run on a minimum of six separate discs (from at least three different tails). Control discs were treated equally (stored, handled, dissected, and snap-frozen) to perform as true unloaded controls.

### RT-PCR

After the treatment period, the discs on either side of the vertebrae being directly loaded (either side of center vertebrae) were dissected from the tails, divided into nucleus pulposus (NP) and annulus fibrosus (AF), flash-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until extraction of total RNA. 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/annular demarcation, and distinct lamellas). 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.<sup>39</sup> Briefly, total RNA was isolated using the Trispin method and quantified using the Sybrgreen assay (Invitrogen). A sample containing 1  $\mu\text{g}$  of RNA was reverse-transcribed using poly-T primers (Omniscript RT kit, Qiagen, Toronto, Ontario). 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 RT-PCR was performed using SYBR green chemistry (SYBR Green Premix, Bio-Rad, Hercules, CA) 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 and then normalized to control sample set.

### TUNEL

In a previous publication<sup>36</sup> using the same tissue culture and handling techniques as this study, a subset of discs (1 per condition) were separated from the bone, embedded in paraffin, sectioned at 8  $\mu\text{m}$ , and mounted on glass slides for 40 $\times$  viewing. A full description of the methodology can be found in Desmoulin *et al.*<sup>36</sup>

**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

### Imparted Mechanics

Raw voltage from the load cell was converted to average peak Newtons of force, and each axis of the accelerometers were converted to average peak *g*'s. Further analysis consisted of converting *g*'s to  $m/s^2$ , integrating the signal twice, and scaling it to mm/s so that strain could be estimated along all axes of the adjacent disc.

### RT-PCR

Data were first analyzed using General Linear Model analysis; however, substantial non-normalities were detected using normal probability plots. Therefore, the analysis was revised using the Kruskal–Wallis nonparametric test. To conduct *post hoc* analyses on the non-normal data, a Box–Cox analysis was performed. In all cases, a transform of  $\lambda = 0.5$  was found to be optimal. The transformed data were then analyzed using ANOVA and Tukey's *post hoc* test. The results of the original Kruskal–Wallis test and the transformed ANOVA test were consistent in all cases, suggesting that the transform was effective in normalizing the data. The transform was only applied for the analysis, not the presentation of the data in the figures. Pairwise comparisons were considered significant at or below the  $P = 0.05$ . All bar graphs plot the mean  $\pm$  standard error.

## RESULTS

### Imparted Mechanics

Table 2 shows that for similar current amplitudes sent to the voice coil, the KKT stylus (which is fixed to the coil) applies a similar force to the tissue sample despite the difference in frequencies (16 and 50–80 Hz). Linear regression quantified the relationship between current and force (Figure 2). Linear equations are plotted for each frequency, the  $R^2$  values are  $>0.862$  for each plot. The accelerations of the measured vertebrae were also similar across current amplitudes despite different frequencies. However, the relative strains along the X- and Z-axes tend to differ over the two frequency levels. At

16 Hz, the relative strains tend to be larger across the same current values than 50 to 80 Hz. The largest shear strain occurred in the Z-axis (3.28% peak strain) which was parallel to the loading axis, with substantially less shear strain in the Y-axis (transverse shear, 0.13% peak strain). Linear strain in the X-axis was measured at 2.56% peak strain.

### RT-PCR

Both the Kruskal–Wallis test and the ANOVA on transformed data indicated that there were significant differences between treatments for aggrecan, collagen type II, and versican ( $P = 0.039$ ,  $0.039$ , and  $0.001$ , respectively; Figure 3), but no significant differences for biglycan, collagen type I, and decorin ( $P = 0.113$ ,  $0.182$ , and  $0.128$ , respectively; Figure 4).

*Post hoc* analysis indicated that aggrecan expression was significantly higher than control at the combined frequencies of 16 + 50 to 80 Hz ( $P = 0.016$ ). Collagen type II expression was significantly different between the 16 Hz and the 16 + 50 to 80 Hz treatments ( $P = 0.0347$ ) but neither was significantly different from control. Versican expression was significantly higher than control at 16 Hz ( $P = 0.0257$ ), 16 Hz was significantly higher than 16 + 50 to 80 Hz ( $P < 0.001$ ), and 16 Hz was significantly higher than 50 to 80 Hz ( $P = 0.0146$ ). No other comparisons were statistically significant at  $P > 0.05$ .

### TUNEL

Results of histologic sections using the same techniques as this study have been published in detail elsewhere<sup>36</sup> but are summarized here. Grossly, the disc itself was observed to be normal with no signs of degenerative changes. TUNEL analysis indicated a mean background apoptosis rate of  $10 \pm 0.7\%$  (mean  $\pm$  standard error). There was no significant difference between controls and frequencies tested ( $P = 0.08$ ), nonloaded and amplitudes tested ( $P = 0.44$ ), or anulus/nucleus ( $P = 0.53$ ).

## DISCUSSION

Vibrations may have beneficial effects on intervertebral disc tissue, although the story to date is confusing. Studies have

**TABLE 2. Imparted Mechanics and Resulting Relative Disc Strain**

Amp (To Actuator)	Frequency (Hz)	Force on Vertebrae (Peak-N) – Ave	SD	Acceleration of Vertebrae (Peak-g) – Ave	SD	Relative Strain of Disk (%) X-Ave	X-SD	Relative Strain of Disk (%) Y-Ave	Y-SD	Relative Strain of Disk (%) Z-Ave	Z-SD
~0.946	16	5.8	1.4	1.17	0.21	0.00	0	0.00	0.00	0.24	0.39
~1.132	16	9.3	2.5	1.77	1.14	0.34	0.56	0.07	0.12	0.55	0.67
~1.522	16	10.6	2.0	2.49	0.81	0.85	1.70	0.00	0.00	0.55	0.67
~1.898	16	12.2	1.9	2.79	0.39	2.56	3.40	0.00	0.00	3.28	3.69
~0.936	50–80	5.5	1.0	0.93	0.15	0.25	0.29	0.10	0.17	0.20	0.24
~1.122	50–80	6.9	1.1	1.27	0.28	0.47	0.53	0.13	0.23	0.57	0.39
~1.484	50–80	11.1	1.7	2.81	0.96	0.23	0.32	0.00	0.00	0.63	0.39
~1.864	50–80	12.2	1.9	3.03	1.09	0.55	0.35	0.00	0.00	0.90	0.39



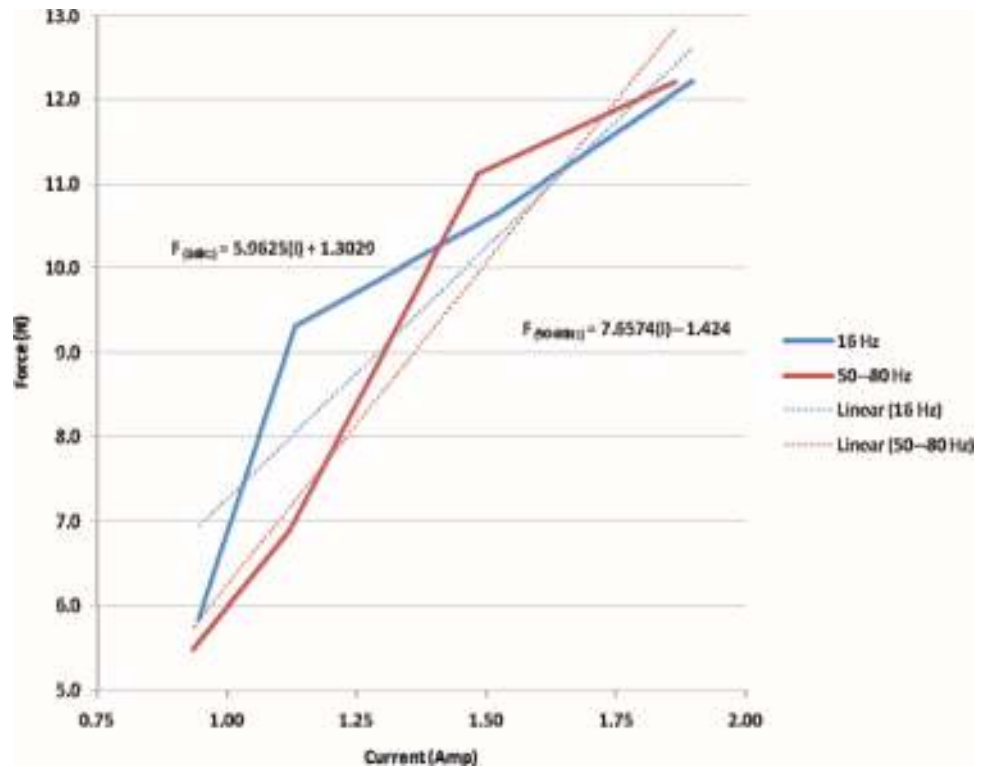


Figure 2. Force (N) versus current (I) chart with plotted linear regression results.

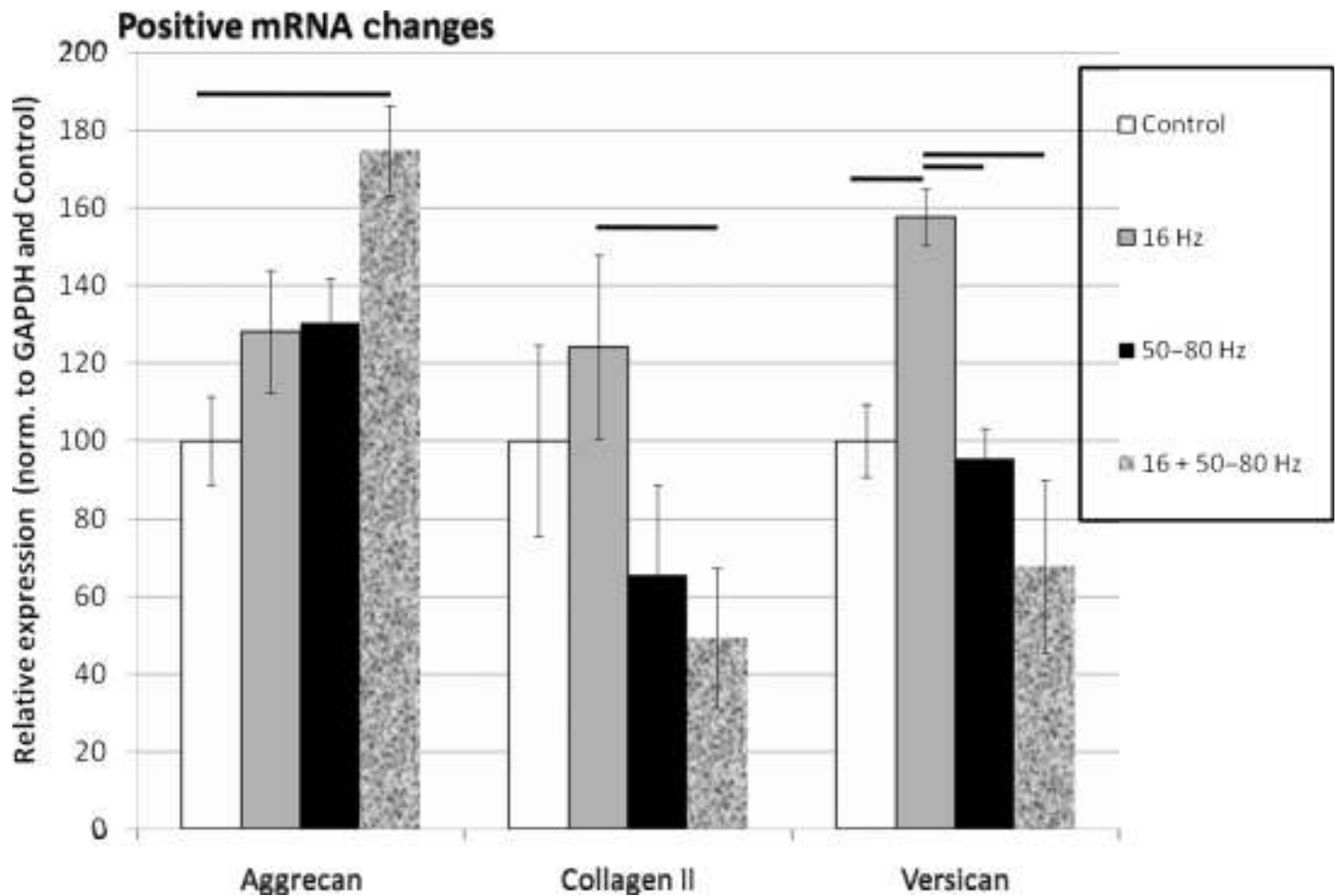
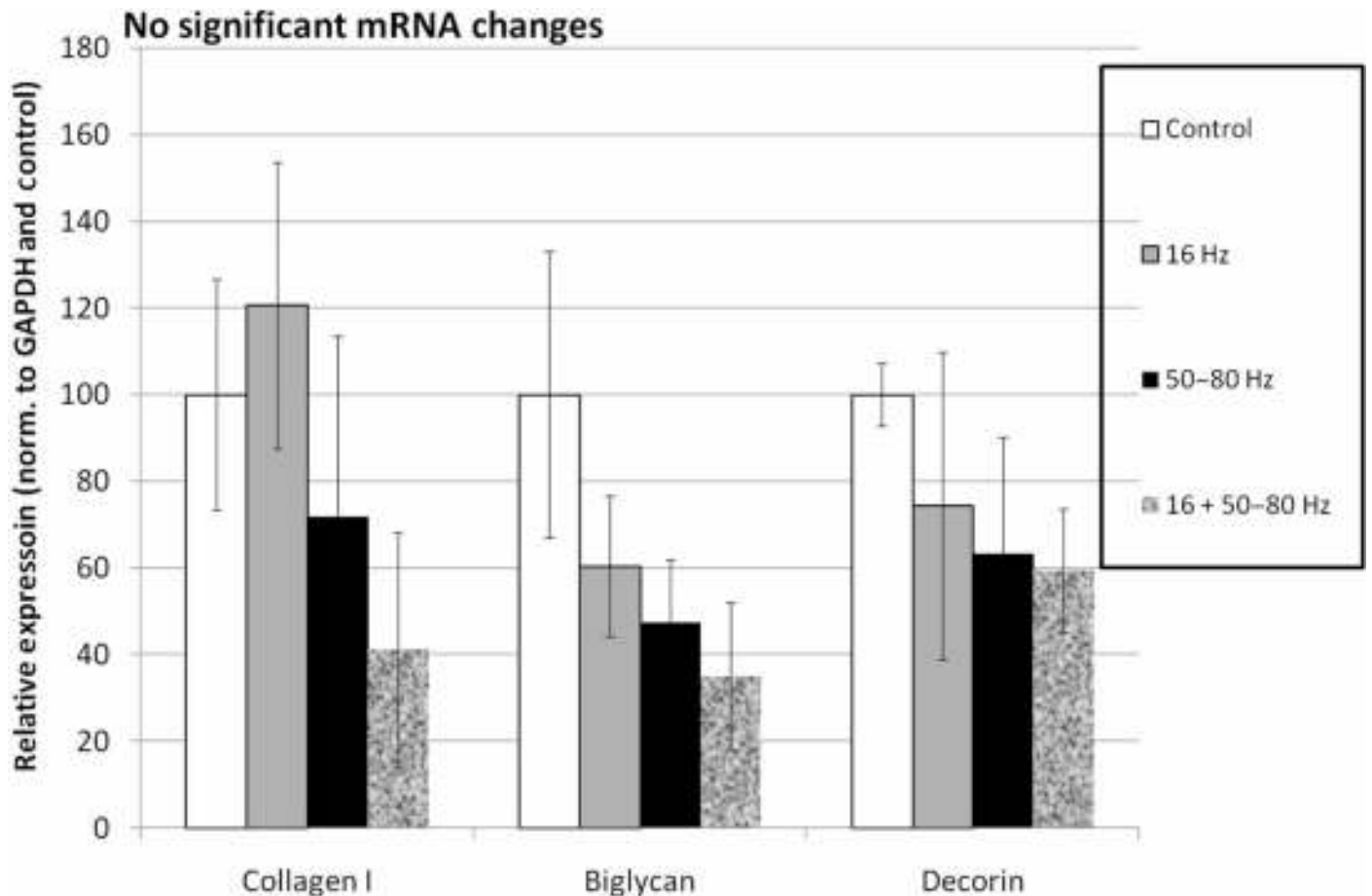


Figure 3. Positive mRNA expression changes included the genes aggrecan, collagen type II, and versican.



**Figure 4.** No significant mRNA expression changes included the genes collagen I, biglycan, and decorin.

variously suggested either an increased risk of disc degeneration with vibrations,<sup>40</sup> no effect,<sup>41</sup> analgesic effect,<sup>7</sup> or even positive effects<sup>36</sup> of vibrations within certain loading parameters. Thus, further investigation of the cell- and tissue-scale processes are warranted.<sup>42</sup>

Evaluating mRNA changes is challenging, because mRNA does not always correlate to functional protein changes. In this study, we accept any statistically significant change as worth attention, regardless of potential “functional change.” Under this definition the current findings indicate that there is potential for this approved clinical tool to beneficially influence gene expression in the IVD under certain loading patterns.

In general, the results presented here indicate that a particular window of vibration may have a positive effect on extracellular matrix gene expression when applied using the KKT device. Aggrecan and versican were above control levels for the specific frequencies and combination of frequencies tested, and there was a statistical suggestion that collagen type II may be affected as well. Aggrecan, collagen type II, and versican are important proteins for disc health and were above control levels for the specific frequencies and combination of frequencies tested.

Aggrecan is a very large proteoglycan (>2800 kDa) with largely mechanical function in the tissue matrix<sup>43,44</sup> but has

been shown to be critical in disc health if absent. Johnson *et al*<sup>45</sup> examined the *in vitro* effects of aggrecan removed from normal human disc tissue and altered aggrecan had on neurite outgrowth. They showed that aggrecan derived from normal IVD inhibited the growth of neurites, but deglycosylated aggrecan, similar to that found in the degenerate IVD, had a reduced inhibitory effect. This suggests that normal aggrecan is an inhibitor of nerve ingrowth into the IVD, and that in degeneration nerve ingrowth may occur as a consequence of changed aggrecan biology. This is important because a constant finding in the analysis of excised painful IVD has been the presence of nerves and blood vessels within the usually aneural and avascular tissues of the IVD.<sup>46</sup>

Collagen type II is the dominant collagen in the nucleus, and although it is clear that disc degeneration is accompanied by loss of proteoglycans, it is still unclear as to the possible changes to collagen II during degeneration.<sup>47-49</sup>

Versican is a large proteoglycan with a molecular weight of approximately 1000 kDa, with functions that are less clear than other large proteoglycans such as aggrecan, but which involve cell adhesion and cell signaling.<sup>50</sup> Interestingly, versican mirrors the decreases in aggrecan gene and protein expression during various stages of disc degeneration.<sup>51</sup>

Further, aggrecan, collagen type II, and versican are highly expressed in the nucleus pulposus of a healthy disc, therefore increased expression for these proteins genes would be expected to correlate to tissue maintenance or repair. In contrast, collagen type I is normally expressed at low levels in healthy nucleus pulposi. Therefore, the combination of nonsignificant changes in expression of collagen type I and increased expression of aggrecan, collagen type II, and versican suggest a potential beneficial effect of the current vibration loading pattern tested with KKT in this study. Further studies will be required to determine the complete positive effect of specific vibration loading patterns.

It is interesting to note that in a previous study<sup>36</sup> aggrecan was not affected by a range of amplitudes, frequencies, or durations of vibration. However, key differences between these two studies are that the previous study applied unconstrained axial vibration, whereas in this study we applied pseudo-constrained shear loading that better represents clinical application with the KKT device application process. This suggests that stylus placement and/or level of disc constraint are additional important aspects affecting disc gene expression.

The current data are insufficient to determine whether the gene expression changes translate into altered protein expression. However, it is certainly clear that the particular loading patterns tested with KKT here positively influence mRNA expression in genes responsible for disc health and seem to avoid increasing expression of genes that control proteins that are normally found in low quantities in a healthy disc. Further study is warranted to determine how these upregulations actually affect the disc.

## ➤ Key Points

- Our previous research using free axial vibration showed that certain loading patterns cause positive (upregulation) mRNA expression in genes responsible for disc health.
- An FDA and Health Canada approved back pain intervention based on vibration can now be evaluated using similar loading patterns for eventual use in patients with degenerative disc disease and discogenic back pain.
- Using the intervention with optimal loading patterns and in a similar orientation as it is used in the clinic on bovine tail segments causes aggrecan, collagen type II, and versican (genes largely responsible for disc health) of the nucleus pulposus to be positively influenced.
- Proteins normally found in low concentration in the nucleus pulposus such as collagen type I were not significantly influenced.
- In combination, these findings suggest that a potential beneficial effect of the current vibration loading pattern tested with the intervention.

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