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The Combined Effects of Limited Nutrition and High-Frequency Loading on Intervertebral Discs With Endplates

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Study Design. Whole ovine caudal intervertebral discs were cultured under simulated-physiologic or high-frequency loading and either sufficient or limited nutrition for 7 days.

Objective. To study the effect of high-frequency loading under sufficient or limited glucose conditions and to investigate the additive effects of load and nutrition on cell survival, gene expression, and cell activity after 7 days of culture.

Summary of Background Data. Limited nutrition and certain mechanical stimuli are generally believed to be etiologic factors for disc degeneration. Although these effects and their interactions have been demonstrated in cell culture, no investigations have been reported in entire discs.

Methods. Discs were maintained in a whole organ culture bioreactor system under simulated-physiologic (0.2 Hz) or high-frequency (10 Hz) loading, in media with either limited (2 g/L) or sufficient (4.5 g/L) glucose concentration. After 7 days, cell viability, gene expression, newly synthesized chondroitin sulfate content, glycosaminoglycan synthesis rate, and disc morphology were assessed after culture and compared with fresh tissue.

Results. Culture under either limited glucose or high-frequency loading conditions led to a significant drop in cell viability. Combined treatment with limited glucose and high-frequency loading resulted in an additive increase in cell death in both the anulus fibrosus and nucleus pulposus and in an increase in MMP13 gene expression.

Conclusion. Supporting in vivo studies and cell culture experiments, high-frequency loading simulating vibration conditions shows detrimental effects on intervertebral disc cells in whole organ culture. The effect on cell viability was exacerbated by limited nutrition culture. However, neither frequency nor limited glucose affected cell metabolism, measured by glycosaminoglycan synthesis rate. Longer culture periods may be required to detect changes at the extracellular matrix level.

Key words: low back pain, intervertebral disc, organ culture, limited nutrition, high-frequency load, cell viability, gene expression. Spine 2010;35:1744–1752

Low back pain (LBP) is the primary cause of disability in the active age group in the Western society, with a lifetime prevalence estimated at 80%. In many cases, LBP is related to intervertebral disc (IVD) degeneration. Although its etiology is still unclear, a potential cause for degenerative changes is diminished nutrition. The disc consists of soft tissue between the bony vertebrae. The cartilaginous endplates (EPs) are adjacent to the gelatinous nucleus pulposus (NP), which is surrounded by the anulus fibrosus (AF), a highly orientated fibrous tissue with a lamellar structure. As the largest avascular structure in the human body, the IVD highly depends on diffusion of nutrients into the disc center. Because of low cell density (4000–9000 cells/mm³), low cell activity and avascular condition, the disc has limited self-repair capabilities. As early as 1931, Beadle observed that nutrition to the disc is mainly supplied via the capillary vascular buds in EPs, and more recent studies have corroborated this finding. During a lifetime, the EPs become less permeable correlating with an increase in disc degeneration, which is believed to be caused by an even further decrease in nutrient supply. Conditions such as smoking, vascular disease, and vascular insufficiency have been associated with higher incidence of disc degeneration and calcification in the cartilaginous EPs. Moreover, occluding the osseous EP openings have been correlated with severity of disc degeneration.

There are also indications that dynamic loading can be deleterious to the disc. Dynamic compressive loading has been shown to lead to degenerative changes to the disc, such as breakdown of the extracellular matrix (ECM). In epidemiologic studies, exposure to whole-body vibration has also been related to LBP. Particularly, vibration in the 4 to 10 Hz range was found to stimulate degenerative processes. This may be due to resonance. The natural vibration frequency of the human body ranges from 3 to 14 Hz, with the higher frequencies representing bending vibration of the upper torso with respect to the lumbar spine. Therefore, the natural frequency of the human body and that of heavy machinery like tractors are similar. In a case control study, Kelsey described a significant association between herniated NP and truck driving with an odds ratio of 4.7. The impact of vibration on disc cells has also been studied in vitro. In monolayer culture, Yamazaki et al cultured rabbit AF cells for 6 days. On the last day, cells were stimulated with a frequency of 6 Hz for 2, 4, 6, and 8 hours. They found that gene expression for ECM mol-
ecules and matrix metalloproteinases was decreased. Alternatively, Kasra et al.\textsuperscript{22} cultured porcine IVD cells in alginate beads under dynamic hydrostatic loading at 1, 3, 5, 8, and 10 Hz with the same amplitude of 1 MPa. They described a drop in DNA amount, impaired protein synthesis, and increased degeneration, at a frequency range of 3 to 8 Hz.

Although the response to specific stimuli, such as nutrition and mechanical stress, can be investigated in detail in isolated cells, removal from their native, highly specialized, extracellular environment doubtlessly affects the cell behavior.\textsuperscript{23,24} Therefore, we aimed to investigate the impact of vibration conditions in an \textit{in vitro} IVD organ culture system, which has not been demonstrated before. We have previously reported that whole IVDs with intact EPs can be maintained viable for up to 3 weeks when cultured under simulated physiologic loading and sufficient nutrition conditions in our IVD organ culture system.\textsuperscript{25} Moreover, limiting nutrition resulted in a decrease in viable cells within days and a new plateau at a lower cell density (50\%) was established and remained stable for up to 3 weeks. This finding corroborates earlier studies and clearly supports the detrimental effects of impaired nutrition on the IVD,\textsuperscript{26} an organ in which, under ideal conditions, a precarious metabolic environment exists.

In this study, we hypothesized that impaired nutrition creates a hostile interstitial environment in which the degenerative processes induced by vibrationally loading on the IVD are amplified. Whole IVDs with EPs were exposed to high-frequency (10 Hz) loading to mimic deleterious vibration, which then was compared with “physiologic” (0.2 Hz) loading. Moreover, the influence of high-frequency load in IVDs cultured under “limited” nutrition conditions was also investigated to reproduce the situation of potentially injurious loading in discs suffering from a compromised nutrition state, \textit{e.g.}, due to EP calcification. This allowed us to reveal potential interactive effects of excessive load and limited nutrition, which are both known etiologic factors in IVD diseases, in a whole organ culture. In contrast to physiologic loading, which is likely to accelerate diffusion of nutrients into the disc, high-frequency load, similar to a static loading situation, may even slow down this process (for review see Iatridis \textit{et al.}\textsuperscript{3}), because the time frame between loading periods is too short to allow significant fluid flow. We, therefore, hypothesized that combining high-frequency loading with nutrient restriction may impair the disc cells in an additive or synergistic manner.

\section*{Materials and Methods}

The procedure for disc harvesting and preparation, as well as the bioreactor used in this study, were described previously.\textsuperscript{28} For this study, discs were harvested from 10 skeletally mature (2–5 years old) Swiss Alpine sheep (\textit{Ovis aries}), which were already being killed for other experiments at our research institute. All prekilling procedures were approved by the Animal Experimentation Commission of the Veterinarian Office and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals. Five caudal discs were prepared from each sheep tail. To exclude disc-level dependency of cell metabolism rates,\textsuperscript{29} the differently cultured as well as day 0 control discs were randomly distributed among the harvested caudal levels. The vertebrae were cut just proximal and distal to EPs with a histologic band saw, and discs were cleaned and loaded in bioreactors as previously described (Figure 1).\textsuperscript{28} Discs were cultured for 7 days either under simulated-physiologic (low) or high-frequency (high) loading, \textit{i.e.}, diurnal axial load (0.2/0.6 MPa, 8/16 hours) with 2 \times 4 hours cyclic load during the 0.6-MPa phase with simulated-physiologic (0.2 Hz; \pm 0.2 MPa) or high-frequency (10 Hz; \pm 0.2 MPa) loading (Figure 2). Discs were cultured in Dulbecco modified Eagle medium containing 10\% fetal calf serum and either limited (lim = 2 g/L) or sufficient (suf = 4.5 g/L) glucose concentration. Because the difference in osmolality between sufficient and limited media was only minor (6.5 mOsm/kg H$_2$O, measured in Dulbecco modified Eagle medium without fetal calf serum), osmolality of the media was not adjusted, avoiding possible interfering effects from substitutes.

The following output parameters were measured to investigate whether limited nutrition and vibration, alone or in com-
tion, have detrimental (catabolic) effects on the disc, resulting in cell death and ECM breakdown: (i) cell viability,25 (ii) expression of anabolic and catabolic genes, (iii) metabolic activity of the disc cells in terms of newly synthesized aggrecan, (iv) glycosaminoglycan (GAG) synthesis rate, (v) collagen content and breakdown, (vi) proteoglycan content, and (vii) disc morphology.

After culture, EPs of discs were removed with a scalpel blade, and the AF and NP were separated with a biopsy punch (diameter = 5 mm). Tissues were then cut into 3 equal parts: one part was used to assess cell viability; the second part was used to assess gene expression; and the last part was further split for collagen breakdown, determination of aggrecan synthesis, GAG synthesis rate, and proteoglycan content. Alternatively, 3 discs were cut in half with a band saw. One half was processed for histology and the other half was used to assess cell viability, gene expression, or aggrecan synthesis.

Cell Viability. Cell viability was determined with 5 μmol/L calcine AM and 1 μmol/L ethidium homodimer-1 (Molecular Probes, Leiden, The Netherlands). Disc tissue was incubated in serum-free medium supplemented with the dyes under free-swelling condition for 2 hours. Stained samples were visualized on an inverted confocal laser scanning microscope (LSM510; Zeiss, Jena, Germany) and analyzed with ImageJ software (http://rsb.info.nih.gov/ij/) as described previously.30

Gene Expression. To assess gene expression, disc tissue samples were flash-frozen in liquid N2, pulverized, and total RNA was extracted with TRI Reagent (Molecular Research Center, Cincinnati, OH), using a modified TRIspin25 method. Reverse transcription was performed using TaqMan RT reagents (Applied Biosystems, Foster City, CA). Expression of selected anabolic and catabolic genes, i.e., aggrecan (ACAN), collagen I (COL1A1), collagen II (COL2A1), ADAMTS4, MMP7, and MMP13, was assessed by real-time reverse transcriptase polymerase chain reaction (ABI GeneAmp 7500, Applied Biosystems), using 18S ribosomal RNA as the endogenous control.28,31 Unpublished sequences are as follows: MMP7, forward primer (5′–3′) GTG GCC AAG GCC TTC AAA and reverse primer (5′–3′) CTT CTT GCA AAG CCA ATC ATG A; and probe, (5′FAM–3′TAMRA) AGC GAA GCA ATC CCA CTG ACG TTT AAG A. Samples with undetectable expression of the target gene were assigned a C, of 45 (maximum number of cycles), representing extremely low mRNA concentration.

Aggrecan Synthesis. Aggrecan synthesis in each tissue sample was assessed by measuring the amount of the chondroitin sulfate 846 (CS846) epitope of the proteoglycan aggrecan, as described previously.25 The monoclonal antibody 846 recognizes a chondroitin sulfate epitope on the largest aggrecan molecules, showing 100% aggregate ability with hyaluronan. The epitope content is barely detectable in normal adult cartilage and discs, and is increased in osteoarthritic cartilage and degenerated discs, and is highest in fetal and neonatal cartilage.32,33 The proteoglycans were extracted in 1 mL of CHAPS buffer (4 M guanidinium chloride in 50 mmol/L sodium acetate and 0.5% 3-(3-cholamidopropyl) diethylammonio-1-propanesulfonate), pH 5.8, with protease inhibitor cocktail, Sigma P8340) and dialyzed. Aliquots of 50 μL were lyophilized and dissolved in buffer supplied with the CS846 Epitope enzyme-linked immunosorbent assay kit (IBEX, Montréal, Canada). Samples were treated and analyzed according to the manufacturer’s instructions, and concentrations of CS846 were normalized to the wet weight of the tissue.

GAG Synthesis Rate by 35S Incorporation. Separate pieces of NP and AF were incubated “free-swelling” for 20 hours in complete medium supplemented with 2.5 Ci/mL 35S-sulfate (Na235SO4; Perkin Elmer, Boston, MA). The tissues were then digested in 1 mL of 0.5 mg/mL proteinase-K solution. Five hundred microliters of both the radio-labeled digests and the medium were loaded onto a PD-10 desalting column (Amer sham, Inc., Cat. no. 17-0851-01) and washed with an eluent solution (pH 7.5) comprising 1 M sodium chlorate, 0.1 M anhydrous sodium sulfate, 0.05 M Tris/hydrochloride, and 0.5% triton X-100. Fractions of 500 μL were collected in 5-mL vials (Simport, Quebec, Canada), combined with 3.5 mL of scintillation fluid (OptiPhase HiSafe3 TM, PerkinElmer Inc., Regensburg, Switzerland), and the activity was counted using a liquid scintillation counter (Wallac 1414 Liquid Scintillation Counter; Perkin Elmer Inc.). The counts per minute were normalized to wet weight of the samples.

Other Biochemical Analyses. The denatured collagen was solubilized with a-chymotrypsin, and the remaining collagen chains were digested with proteinase K33. Percent of denatured and total collagen were assessed by determination of the OH-proline content, in their respective digests.34,35 Proteoglycan amount of proteinase K-digested tissue and of culture media was measured with the dimethylmethylene blue dye assay.33-35 Total water retained in the tissue was also determined.

Histology. For histology, specimens were fixed in 4% buffered formaldehyde, decalified in ethylenediaminetetraacetic acid, and embedded in paraffin. Sagittal sections (6-μm thick) were stained with safranin-O/fast green. Gene expression and biochemical data were normalized to the values of corresponding day 0 control discs.

Statistical Analyses. For statistical analyses, univariate GLM with subsequent pairwise post hoc testing was performed (Fisher LSD and Games-Howell). For all statistical analyses, a P-value <0.05 was considered significant.
**Results**

The median (interquartile range) values of cell viability in fresh discs was 86.02% (84.36–89.89) in AF and 92.24% (90.75–95.97) in NP. For discs cultured in sufficient media under simulated-physiologic (suf/low) conditions, the viability was maintained (AF: 85.35% [81.98–94.72], NP: 89.77% [84.46–92.74]), whereas culturing under limited condition or high-frequency load resulted in decreased cell viability (suf/high: AF: 61.87% [46.52–79.24], NP: 55.04% [48.39–66.16]; lim/low: AF: 65.78% [49.74–73.97], NP: 75.84% [70.90–82.15]). In the NP, high-frequency load had a greater impact on the cell death than limiting nutrition. Combined culture under limited glucose condition and high-frequency load had an additive increase in cell death (lim/high: AF: 39.63% [33.67–49.53], NP: 57.61% [42.91–62.80]; Figure 3).

Gene expression analysis revealed an increase in MMP13 mRNA that was significant for the NP in discs cultured under lim/high conditions (NP: \( P = 0.046 \); AF: \( P = 0.064 \) vs. suf/low). For the other genes, no differences were obtained. Notably, in the NP, MMP7 seemed to be slightly up-regulated when cultured under lim/high conditions; however, this did not reach significance (Figure 4). Aggrecan synthesis was not significantly different between discs cultured in different conditions, neither for AF nor for NP tissue (Figure 5).

GAG synthesis rate by \(^{35}S\) incorporation did not reveal any changes between groups. Neither nutrition nor loading frequency had a noticeable effect on GAG synthesis rates after 7 days of culture, although for the AF, \(^{35}S\) incorporation tended to be slightly increased when cultured under limited nutrition (data not shown). Similarly, denatured collagen, water content, GAG content in tissue, or GAG released in media did not reveal any significant differences between culture conditions.

Morphology of the discs could be maintained during the culture. Analyses of the safranin-O/fast green stain revealed neither rupture of the AF nor cracks in the EP after any culture condition. However, when cultured under high-frequency load, the AF bulged slightly outward compared with fresh discs or discs cultured under lower simulated-physiologic loading frequencies (Figure 6).

**Discussion**

This study is based on previous work in which we cultured caudal ovine discs in an organ culture system with simulated-physiologic loading and media containing sufficient or limited concentration of glucose. In that study, cell viability could be maintained up to 3 weeks if adequate glucose was provided. Although decreasing glucose concentration did cause substantial reduction in living cells, it was not sufficient to provoke other indications for disc degeneration at the gene expression and protein levels during 7 and 21 days of culture. In this study, in addition to limited nutrition, the impact of high-frequency (10 Hz) load was investigated. Exposure to a frequency of \( \sim 10 \) Hz can be experienced when flying in a helicopter and is similar to the natural vibration frequency in a human body.\(^{18}\) In a questionnaire survey Bongers et al\(^{36}\) investigated the prevalence of LBP of helicopter pilots and found that it correlated with total flight time (odds ratio, 13.4). In a laboratory simulation, Pope et al\(^{18}\) reproduced the environment of a helicopter and measured a loss of comfort of the volunteers already after a 2-hours exposure. We, therefore, hypothesized that 7-day culture under high-frequency load would induce metabolic changes in our system. Disc degeneration is caused by multiple factors. These can be genetic or epigenetic such as age-related or physiologic or linked to living or occupational circumstances. Therefore, we specifically aimed to investigate the single and combined...
effect of high-frequency load as an environmental factor and limited nutrition as an intrinsic factor, resulting for example from EP calcification. In particular, vibration-like loading may reduce the diffusion of nutrients and, at the same time, enhance the accumulation of acidic metabolites within the disc. If nutrient supply is already diminished, this may lead to accelerated cell death. Interestingly, high-frequency compression had a greater im-

Figure 4. Relative gene expression of 3 anabolic and 3 catabolic genes after 7 days of culture. A, Anulus fibrosus (AF) and B, nucleus pulposus (NP). Reverse transcriptase polymerase chain reaction data were normalized to house-keeping gene (18S rRNA) and are presented relative to day 0 (d0) fresh control. Compared with suf/low-cultured discs, MMP13 was more highly expressed in the stressed discs under lim/high conditions (+0.05 < P < 0.065; ++ is P < 0.05) and * mark outliers.
impact on the NP cells than limiting nutrition, whereas the AF cells were affected to a similar extent by both stimuli. This outcome essentially results from a less-pronounced effect of nutrition deficiency on the NP than AF cells. NP cells are physiologically exposed to harsh conditions and may, therefore, be more resistant to nutritional challenges than AF cells.37,38 Detrimental load, however, equally affected both AF and NP cells. The combined parameters (lim/high) increased the cell death in an additive manner, resulting in lowest viability in the AF of lim/high-cultured discs. These data, extrapolated to the human situation, could mean that subjects with IVD nutrition problems, which are additionally exposed to high-frequency vibration sources (~10 Hz), might be more likely to suffer from disc degeneration with respect to increased cell death. Although our data may suggest that impaired nutrition and excess load may separately contribute to changes in the IVD cell viability, it is im-

Figure 5. Box plots of CS846 expression relative to day 0 (d0). Neither in anulus fibrosus (AF) nor in nucleus pulposus (NP) significant differences were detected between culture conditions or compared with fresh discs. * marks outliers.

Figure 6. Sections of safranin-O/fast green stains of discs before and after 7 days of culture. A, day 0 (d0); B, suf/low; C, suf/high; D, lim/low; and E, lim/high.
important to note that these parameters cannot be regarded as completely independent. It has been shown in animal models that vibration itself may lead to compromised nutrition.\textsuperscript{39,40} Hence, when combined with limited glucose supply, vibration may affect the disc cells in a synergistic manner. Analysis of additional parameters addressing the nutrition state of the disc cells will be required to elucidate the damaging mechanisms of vibration stress more in depth.

Although cell viability assays can evaluate the survival rate of cells, they may not assess their metabolic activity. The GAG synthesis rates we obtained for fresh discs and discs cultured under sufficient nutrition and low frequency are in line with those reported by other investigators, who also documented higher values in the NP compared with the AF.\textsuperscript{26,29,37,41} In addition, on comparing the different culture conditions, no changes were obtained for the NP after 7 days. According to the heightened nutrient sensitivity of the AF with respect to cell viability, the slight increase in \textsuperscript{35}S incorporation in the limited cultured discs could indicate a first metabolic response of the challenged discs. Because our experimental set-up did not allow us to measure the sulfate incorporation during culture, the \textsuperscript{35}S incorporation was determined while the tissue was in free-swelling conditions, which might affect synthesis rates. Thus, our comparisons were based only on relative values (between groups). Moreover, we additionally measured the amount of newly synthesized aggrecan by CS846 quantification. \textit{In vitro} studies have shown a direct correlation of the content of this epitope in cartilage to aggrecan synthesis rate measured by \textsuperscript{35}S incorporation into GAG.\textsuperscript{42,43} The half-life of CS846 has been estimated to be 15 to 20 hours.\textsuperscript{44} Hence, it may be considered to be representative of a newly synthesized subpopulation of aggrecan molecules during culture time.\textsuperscript{35} Still, corresponding to the \textsuperscript{35}S incorporation, no significant differences could be obtained between culture conditions (Figure 5).

With the up-regulation of MMP13, the discs responded directly at the gene expression level to the additive culture (lim/high). Remarkably, other catabolic genes, \textit{i.e.}, ADAMTS4 and MMP7 were not up-regulated after the 7-day culture. During IVD degeneration, the expression of several matrix-metalloproteinases increases, including MMP1, 3, 7, 9 and, 13, all known to degrade many of the main matrix components.\textsuperscript{45–47} It has been shown that of these MMPs, MMP7 and 13 (whose predominant targets are collagen type II and aggrecan) are most highly expressed within degenerate discs, particularly in the NP.\textsuperscript{45,46} However, because MMPs are secreted in latent form (pro-MMPs) and their activation is regulated by specific tissue inhibitors of metalloproteinases, which inhibit certain particular MMPs,\textsuperscript{48} the pro- and activated forms are indistinguishable from each other by means of relative gene expression analysis. Le Maitre \textit{et al}.\textsuperscript{46} reported that MMP7 is more resistant to tissue inhibitor of metalloproteinase-1 and -2 deactivation and, therefore, might have a key function in terms of disc matrix remodeling. As a result, MMP7 may be activated in the stressed disc at the post-transcriptional or even post-translational level. Hence, inactive pro-MMP7 present in the nondegenerated disc may be converted to the active isoform in the stressed discs, which cannot be detected at the mRNA expression level. In contrast, MMP13 is hardly expressed in non-degenerated discs but increases with degeneration,\textsuperscript{45} which is in line with our observed increase in MMP13 expression and points to an early sign for a response of the stressed discs.

Because of the clear increase in cell death after the “stressing” culture, one could assume a greater response in gene expression and ECM turnover by the disc cells. The study by Korecki \textit{et al}.\textsuperscript{49} supports our findings in this respect. They cultured young and mature bovine disc cells in 3-dimensional alginate, and exposed them to frequency load for 7 days (0.1, 1, and 3 Hz compared with free swelling). Exposure of mature bovine disc cells to frequency load did not result in changes, neither in DNA nor in GAG content. Responses in gene expression were only minor for AF cells, whereas for NP cells, no effect of loading was observed. Similar findings were observed by Wuertz \textit{et al}.\textsuperscript{50} in an \textit{in vivo} rat tail model. Even after 8 weeks with daily loading for 8 hours at 1 MPa and 1 Hz frequency, only a mild degenerative shift was observed when compared with unloaded discs.

Although changes were detected at the cell viability and gene expression level, our harsh culture conditions of limiting nutrition combined with a frequency of 10 Hz are apparently not sufficient to induce disc degeneration at the protein level during a short-term whole organ culture. With \textasciitilde{}2 \times 10^6 cells/g wet weight in fresh discs, cell density is relatively low, limiting cell-to-cell signaling; consequently, the single cell cannot sense that the surrounding cell is stressed. Moreover, potential alterations in the ECM composition or turnover might be below the detection limit of the applied biochemical assays. It has to be considered that relatively few cells are responsible for a comparably immense amount of ECM. Assuming that cell viability decreases from \textasciitilde{}100\% to \textasciitilde{}50\% in a biomechanically stressed disc, the differences in matrix production would be hardly detectable, even if the remaining cells extensively increased their metabolic activity.

The interanimal variation was minimized because only study animals from the same breeder were used. However, these sheep were between 2 and 5 years of age, which may imply age-related differences, with a relatively small sample size. Nevertheless, it is conceivable that if the cells were given more time to respond to the changing conditions, \textit{e.g.}, by increasing the culture time, one could expect a stronger response also on the ECM level. Therefore, further experiments will investigate effects of extreme high-frequency (10 Hz) loading and varying nutrition in a midterm culture (\textit{i.e.}, 3-week culture).
Current treatment strategies for discogenic pain are generally limited to spinal fusion or mechanic implants, and little is known about biologic regeneration strategies, e.g., cell or growth factor therapy. In vitro models for investigating potential preventive or regenerative therapies are rare even though it is desirable to test the applicability of new treatment strategies in an environment simulating in vivo conditions. In this context, our in vitro whole organ IVD culture system may be considered as a valuable tool not only for investigating potential mechanisms leading to the onset of IVD degeneration (excessive loading and limited nutrition), but also for evaluating new regenerative strategies, e.g., by monitoring the fate (survival and differentiation) of injected mesenchymal stem cells or the impact of growth factor therapy.

**Key Points**
- Both high-frequency loading and limited nutrition reduced cell viability in discs cultured in a whole organ bioreactor for 7 days. Combining both parameters increased cell death furthering an additive manner.
- Compensatory mechanisms of the remaining disc cells, i.e., up-regulation of anabolic gene expression and increased proteoglycan synthesis along with increased cell activity, were not observed under either high frequency or limited glucose conditions.
- To promote in vitro disc degeneration at the extracellular matrix level, longer culture periods may be required.

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**References**


