Original Article

Effect of Static Load on the Nucleus Pulposus of Rabbit Intervertebral Disc Motion Segment in *Ex vivo* Organ Culture

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Abstract

Background: The development of mechanically active culture systems helps increase the understanding of the role of mechanical stress in intervertebral disc (IVD) degeneration. Motion segment cultures allow for preservation of the native IVD structure, and adjacent vertebral bodies facilitate the application and control of mechanical loads. The purpose of this study was to establish loading and organ culture methods for rabbit IVD motion segments to study the effect of static load on the whole disc organ.
Methods: IVD motion segments were harvested from rabbit lumbar spines and cultured in no-loading 6-well plates (control conditions) or custom-made apparatuses under a constant, compressive load (3 kg, 0.5 MPa) for up to 14 days. Tissue integrity, matrix synthesis, and the matrix gene expression profile were assessed after 3, 7, and 14 days of culturing and compared with those of fresh tissues.
Results: The results showed that *ex vivo* culturing of motion segments preserved tissue integrity under no-loading conditions for 14 days whereas the static load gradually destroyed the morphology after 3 days. Proteoglycan contents were decreased under both conditions, with a more obvious decrease under static load, and proteoglycan gene expression was also downregulated. However, under static load, immunohistochemical staining intensity and collagen Type II alpha 1 (*COL2A1*) gene expression were significantly enhanced (61.54 ± 5.91, $P = 0.035$) and upregulated (1.195 ± 0.040, $P = 0.000$), respectively, compared with those in the controls ($P < 0.05$). In contrast, under constant compression, these trends were reversed. Our initial results indicated that short-term static load stimulated the synthesis of collagen Type II alpha 1; however, sustained constant compression led to progressive degeneration and specifically to a decreased proteoglycan content.
Conclusions: A loading and organ culture system for *ex vivo* rabbit IVD motion segments was developed. Using this system, we were able to study the effects of mechanical stimulation on the biology of IVDs, as well as the pathomechanics of IVD degeneration.

Key words: Disc Degeneration; Intervertebral Disc; Motion Segment; Organ Culture; Static Load

INTRODUCTION

Evidence of a link between degenerative intervertebral disc (IVD) and low back pain (LBP) is mounting.[1] Currently, the treatment of diseases related to IVD often involves surgical intervention or long-term rehabilitation therapy. The goals of biological therapy are to prevent or delay IVD degeneration and to alleviate its symptoms by promoting tissue repair. To better prevent and treat LBP, a detailed understanding of the mechanisms of IVD degeneration is necessary.

The comprehensive mechanisms and related biological and mechanical pathways of IVD degeneration remain poorly understood even though LBP is a common clinical condition.[2,3] Biomechanics and IVD degeneration are closely related, and many studies have shown that mechanical loading is one of the major factors leading to IVD.[4,5] An epidemiological survey has also shown that mechanical loading is a risk factor for LBP.[6,7] However, weight-bearing mechanical loading is the primary function of IVDs, and it occurs in the natural environment.

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Mechanical loading also stimulates IVD cells; this stimulation is likely the underlying factor for the maintenance of cartilage cell activity.[8,9] To better understand the mechanisms of IVD degeneration, an in-depth understanding of its cause and effect relationship with biomechanics is required. Moreover, the establishment of an ideal experimental model might facilitate future-related studies.

However, investigating the complex metabolism and signaling cascades that occur in IVDs is difficult using *in vivo* models because of the lack of close control and difficulties with monitoring.[10] In contrast, *ex vivo* culturing systems are more appealing because they allow for better control of biochemical and biomechanical factors.[1] Therefore, *ex vivo* organ models represent an experimental basis to observe the responses and changes of IVD tissues to specific external stimuli,[1,9-22] such as mechanical loads[23,24] or biologics.[25,26] Thus, an IVD motion segment was established and maintained in an organ culture,[27-29] and it included the whole IVD organ and adjacent vertebral bodies (VBs). The motion segment model has certain advantages. First, it enables the *ex vivo* organ model to be more reflective of the true conditions. Second, the adjoining VBs (as rigid fixtures) facilitate the application and control of complex mechanical loads.[27]

This study compared rabbit IVD motion segments cultured in custom-made apparatuses under static load between control tissues and fresh tissues. The tissue integrity, matrix synthesis, and matrix gene expression profile were assessed to determine the influence of static load on the IVDs and to increase understanding of these conditions so that the effects of biomechanics on the IVDs can be better controlled.

METHODS

Ethics statement

All of the experimental procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals of the China Academy of Chinese Medical Sciences, Beijing, China. The experimental animals included 14 18-week-old (3.0 kg) male New Zealand White rabbits. These animals were used in accordance with protocols that were approved by the Animal Ethics Committee of the Institute of Basic Theory for Chinese Medicine, China Academy of Chinese Medical Sciences (Approval No. 20141002). The animals were anesthetized with pentobarbital (100 mg/kg). Five minutes before death, 25,000 IU heparin was administered through the ear vein, and the lumbar spine was harvested under sterile conditions immediately after euthanasia via CO_2 asphyxiation.

Intervertebral disc motion segment culture method

IVD motion segments were obtained from the 14 rabbits. Soft tissues and posterior elements of the spine were removed, and the motion segment models were dissected from consecutive levels (n = 4/animal) consisting of the whole disc organ, which included the vertebral end plates (EPs), annulus fibrous (AF), and nucleus pulposus (NP) with adjacent VBs. The lengths of the IVD motion segments ranged from 280 to 310 mm, and the diameters of the IVDs ranged from 110 to 140 mm. Any debris on the cut surfaces was washed with phosphate-buffered saline using a syringe with an 18-gauge needle; all of the IVDs were rinsed for 2 min in Hanks' Balanced Salt Solution containing heparin and 10% penicillin-streptomycin.

A total of 28 IVD motion segments were maintained in 6-well plates without loading (controls), and the other 28 IVD motion segments were kept in custom-made apparatuses under a constant compressive load (3 kg, 0.5 MPa). The 6-well plates and apparatuses were maintained in an incubator under standard culture conditions (37°C, 5% CO_2; [Supplementary Figure 1]).

The specimens were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA), 50 mg/ml L-ascorbate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml Fungizone. NaCl was added to the DMEM to raise the osmolarity to 410 mOsm/kg. The media were changed every 2 days. After 0, 3, 7, and 14 days, seven IVDs were assessed by hematoxylin and eosin (HE) staining, Alcian blue/periodic acid-Schiff reaction (AB/PAS), and collagen Type II alpha 1 immunohistochemistry (IHC), and real-time polymerase chain reaction (PCR) was performed to examine the matrix gene expression profile.

Loading and culturing apparatuses for the intervertebral disc motion segments

The apparatuses were designed for the *ex vivo* culturing of IVD motion segments while simultaneously providing static axial compression. They comprised two components: A loading frame and a culture chamber.

The loading frame provided a static load through the use of weights on a mobile loading plate that could be moved along the optical axis. Because compression can cause the IVD heights to change slightly, the mobile weight ensured that the full load was being consistently applied to the specimen. A gland was fixed to the bottom of the loading plate, through which the weight was applied to the model. A gland also covered the top of the chamber to ensure that the culture environment was closed [Supplementary Figure 2].

A disposable silicon tube was inserted at the bottom of culture chamber, and it functioned as an outlet for media changing; the other end of the tube was plugged. A syringe was used for changing the media, and the manipulations were performed on an aseptic operation table. Moreover, the tubing and needle were changed with each use to ensure for sterility of the culture [Supplementary Figure 2].

Based on the characteristics of the spinal motion segment, the device was arranged on the top and base pedestals. Three jackscrews were inserted through the pedestal to fix one side of the VBs, allowing the motion segment to remain in an upright state so that the compression was always vertically loaded on the surfaces of the IVDs [Supplementary Figures 2 and 3].

All of the components could be disassembled from the device, and the materials were resistant to deformation and decomposition by disinfection treatments (e.g., high temperature and high pressure). Moreover, the whole apparatus was sufficiently small; thus, multiple loading stations could be placed in the incubator at the same time [Supplementary Figure 1; Patent number: ZL 201420568511.9].

Histological analysis

The samples were fixed with a 10% buffered neutral formalin solution for up to 2 days, decalcified in ethylenediaminetetraacetic acid, and then embedded in paraffin. Midsagittal sections were cut with thicknesses of 4–6 μm. The sections were deparaffinized in xylene, rehydrated with graded ethanol, and stained with HE. Then, they were viewed by a light microscope.

AB/PAS staining was used to detect synthesized proteoglycans in the IVD tissues.

Collagen Type II alpha 1 immunohistochemistry

For detection of the NP extracellular matrix (ECM), a collagen Type II alpha 1 monoclonal antibody (Sigma, USA) was used for IHC analyses. In brief, at each harvesting time point, 4–6 μm paraffin sections of three samples from each group were dewaxed, rehydrated, and then blocked with hydrogen peroxide as an endogenous peroxidase. Next, the sections were washed in dH_2O and treated with 1 of 2 enzyme antigen retrieval reagents for 20 min at 37°C. They were subsequently washed again, and nonspecific binding sites were blocked at room temperature for 45 min with 20% w/v goat serum. Then, the sections were incubated with a mouse monoclonal primary antibody against collagen Type II alpha 1 (Sigma-Aldrich, Bale, Switzerland) diluted 1:20 overnight at 4°C. Negative controls were used for which the primary antibody was replaced with an equal concentration of mouse IgG (Sigma). After the sections were again washed, those being assessed for collagen Type II alpha 1 were incubated with biotinylated rabbit anti-mouse antiserum (Sigma) diluted 1:400 for 30 min at room temperature. The secondary antibody binding was visualized using the streptavidin-biotin complex technique with a 3,3’-diaminobenzidine tetrahydrochloride solution as the colored reaction product. The sections were counterstained with hematoxylin, dehydrated, and mounted. An NIS-Elements D2.30 image analysis system (Nikon, Tokyo, Japan) was used for semiquantitative analysis of collagen Type II alpha 1.

Real-time polymerase chain reaction analysis

Total RNA from the NP was analyzed for the expression of *ECM* genes. In brief, the AF was cut with a blade, and the NP tissue was removed using a micro curette and immediately frozen in liquid nitrogen at each harvesting time point. Tissues from 3 IVDs were pooled to ensure that a sufficient quantity of tissues was obtained for RNA isolation,[28,30,31] which was performed using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Primers for the rabbit glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *ECM* genes were custom designed using Primer 5.0 software (Applied Biosystems, Foster City, CA, USA) [Table 1].

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using an SYBR Green One-Step qRT-PCR kit (Kapa Biosystems, USA). The final qRT-PCR results were obtained using the comparative C_T method with the following equation:

Fold change = $2^{-\triangle\triangle Ct}$, where $-_{\triangle\triangle}Ct = (Ct_{target} - Ct_{reference})_{control} - (Ct_{target} - Ct_{reference})_{culture}$ (reference: mean Ct of *GAPDH*; control: day 0; and culture: day 3, 7, 14, or 21).

Statistical analyses

Statistical analyses, including analysis of variance (ANOVA) and *post hoc* pairwise comparisons, were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL, USA). The between-group data were compared using the independent-samples *t*-test. All of the results are expressed as the mean ± standard deviation (SD). Statistical significance was set at $P < 0.05$.

Results

Histological analysis

Before culturing, HE staining showed that the central integrated NP contained abundant cells and matrix material. In addition, the AF was arranged in concentric circles with a clear hierarchy and clear boundaries between the NP and the cartilage EP at the upper and lower ends. The section staining did not reveal any major structural change in the controls at 3 or 7 days compared with the fresh samples. The tissue structure remained intact for up to 14 days although a partial separation between the NP and AF was observed. Under static load, the integrity of the tissue was maintained for 3 days. Culturing for 7 days led to a decrease in the number of NP cells and their dispersal, as well as partial laceration of the AF. Culturing for 14 days led to loss of the tight concentric architecture of the AF and separation of the NP; this change was more obvious under static load than under the control conditions [Figure 1].

The specimens were stained with AB/PAS to observe changes in the proteoglycan content.[10] The staining within the matrix and in the immediate pericellular areas indicated the presence of proteoglycans. After 7 days, a marked loss of extracellular proteoglycans was observed. Under static load, the proteoglycan content decreased and was unevenly distributed by 7 days. Moreover, by 14 days, the number of cells in the NP was obviously decreased, although no further decrease in staining was observed, suggesting a reduction in biosynthetic activity [Figure 2].

Immunohistochemistry

Immunoreactive collagen Type II alpha 1 was observed

Table 1: Details for the primers for the target and reference genes used in quantitative real-time PCR

Gene	Primers	Primer Sequence	bp
GAPDH	tuzi-GAPDH-F	CGAGACACGATGGTGAAGGT	131
	tuzi-GAPDH-R	ATGTAGTGGAGGTCAATGAATGG	
Tu-COL2A1	Tu-COL2A1-F	ATGGCGGCTTCCACTTCA	92
	Tu-COL2A1-R	CTCAGTGGACAGCAGGCG	
Tu-agg	Tu-agg-F	TTACCACCTACCCTTCACCTG	90
	Tu-agg-R	TTCTTCTGTCCAAAGGTCCTG	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; *COL2A1*: Collagen Type II alpha 1; *agg*: Aggrecan; F: Forward; R: Reverse; PCR: Polymerase chain reaction.

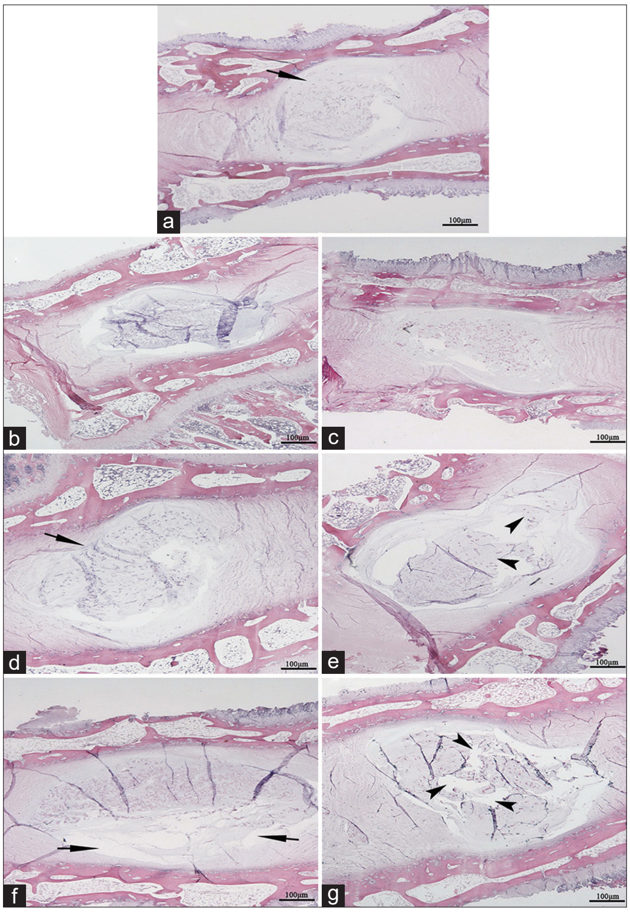

Figure 1: Hematoxylin and eosin staining of IVD midsagittal slices at each time point under control and under static load conditions (original magnification, ×10). (a) Fresh tissue; (b, d, and f) tissue histological observations of organ controls on days 3, 7, and 14, respectively; (c, e, and g) histological observations of tissues under static load on days 3, 7, and 14, respectively. In the controls, the tissue structure remained intact up (a and d, arrows) to 14 days although a partial separation between the NP and AF appeared (f, arrows). Under static load, culturing to 7 days led to a decrease in the number of NP cells and their dispersal (e, arrowheads). Culturing for 14 days led to the loss of the tight concentric architecture of the AF and separation of the NP (g, arrowheads), and this change was more obvious under the static load than that in the control condition. IVD: Intervertebral disc; AF: Annulus fibrous; NP: Nucleus pulposus.

within the matrix of the NP in the controls and tissues cultured in the apparatuses at all times. The staining intensity remained consistent by 14 days in the controls, and no significant difference was observed between the control and fresh tissues ($P = 0.896$).

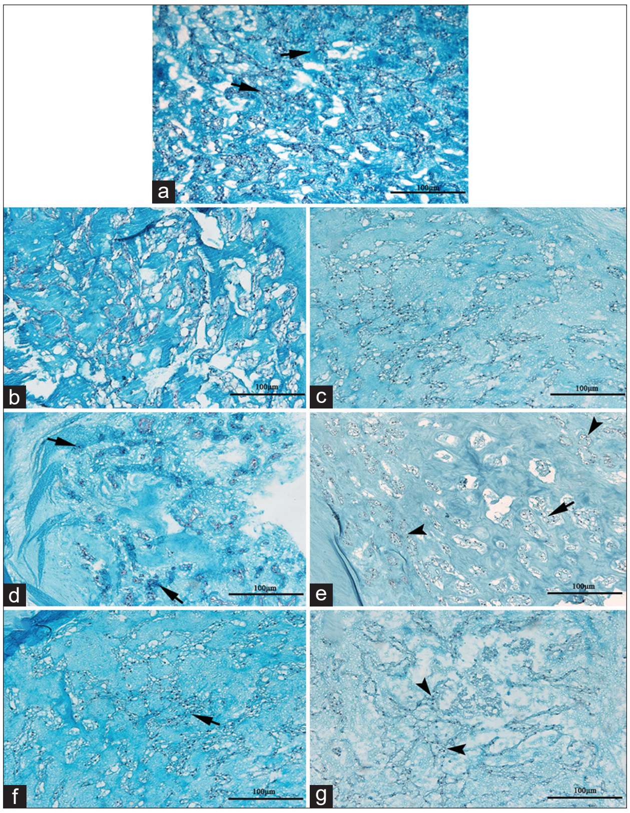

Figure 2: AB/PAS staining of proteoglycans in the NP at each time point (original magnification, ×200). (a) Fresh tissue; (b, d, and f) AB/PAS staining of organ control tissue on days 3, 7, and 14, respectively; (c, e, and g) AB/PAS staining of tissues under static load on days 3, 7, and 14, respectively. The staining within the matrix and in the immediate pericellular areas indicated the presence of proteoglycans (a, arrows). After 7 days, a marked loss of extracellular proteoglycans was observed (d, arrows). (e) Under static load, by 7 days, the proteoglycan content decreased (arrow) and was unevenly distributed (arrowheads). By 14 days, the number of cells in the NP obviously decreased (g, arrowheads). AB/PAS: Alcian blue and periodic acid-Schiff; NP: Nucleus pulposus.

The samples under static load showed a significant increase in IHC intensity during the initial 3 days, and significant differences were observed between the controls and fresh tissues ($P < 0.05$). However, the staining was obviously reduced after 3 days, and by 14 days, it was significantly decreased ($P < 0.05$) and significantly different from that observed in the controls ($P < 0.001$) [Figures 3, 4 and Table 2].

Extracellular matrix gene expression

qRT-PCR revealed marked downregulation of aggrecan gene (*Agg*) expression in the controls after 7 days compared with the fresh tissues whereas it was significantly downregulated under static load after day 0 compared with fresh tissues and controls [Figure 5 and Table 3]. In contrast, collagen Type II alpha 1 (*COL2A1*) expression was upregulated under static load on day 3, but tended to be downregulated under the control conditions; this difference was statistically significant. However, *COL2A1* expression subsequently decreased rapidly to below detectable levels under static load until day 14, but it was not significantly downregulated under the control conditions after 3 days; further, statistically significant differences were observed on days 7 and 14 [Figure 5 and Table 4].

DISCUSSION

The initial objective of this study was to investigate the effect of static load on the whole IVD motion segment in culture

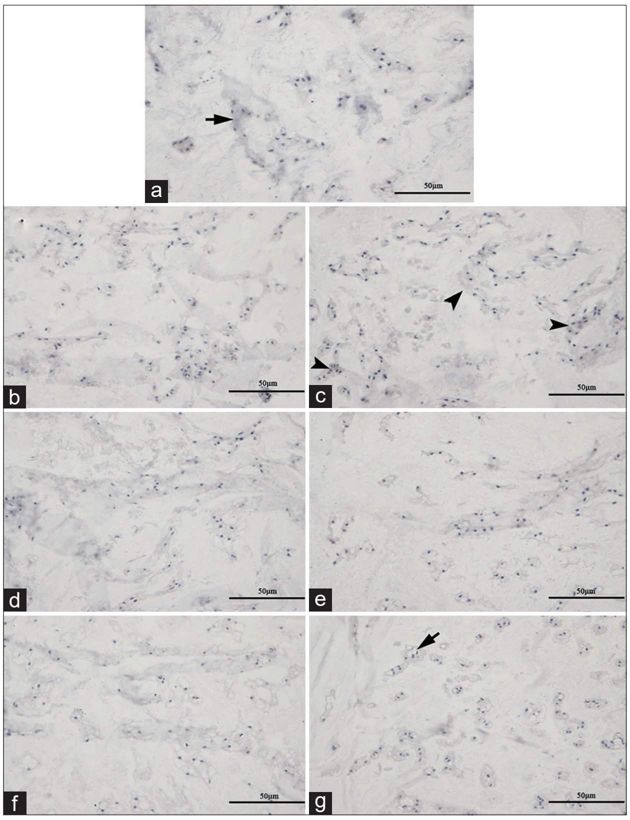

Figure 3: Collagen Type II alpha 1 IHC staining of the NP at each time point (original magnification, ×400). (a) Fresh tissue; (b, d, and f) tissue collagen Type II alpha 1 staining of organ controls at days 3, 7, and 14, respectively; (c, e, and g) collagen Type II alpha 1 staining of tissues under static load at days 3, 7, and 14, respectively. The intensity of staining remained constant by 14 days in the controls, and no significant difference was observed between the controls and fresh tissue (a, arrow). The samples under static load showed a significant enhancement in IHC intensity for the initial 3 days (c, arrowheads); however, the staining was obviously reduced after 3 days, by 14 days (g), the staining was significantly decreased (arrow) and significantly different from the controls. IHC: Immunohistochemistry; NP: Nucleus pulposus.

Table 2: Collagen Type II alpha 1 IHC staining intensity of the NP during organ culture

Culture time point	Controls	Static load	*t*	*P*
Day 0	47.64 ± 8.33	43.75 ± 7.05	0.798	0.448
Day 3	44.74 ± 3.26	61.54 ± 5.91*‡	−2.593	0.035
Day 7	43.47 ± 3.88	22.97 ± 5.03*†‡	2.470	0.039
Day 14	44.58 ± 5.02	30.43 ± 2.13*†‡	5.811	<0.001
F	0.198	20.417	–	–
P	0.896	0.000	–	–

Values are given as the mean ± SD. *The significance of differences between culture time point and fresh (day 0); †The significance of differences between culture time point and day 3; ‡The significance of differences between static load and controls. IHC: Immunohistochemistry; NP: Nucleus pulposus; SD: Standard deviation, –: Not applicable.

Table 3: *Agg* gene expression quantified by real-time PCR during organ culture

Culture time point	Controls	Static load	*t*	*P*
Day 0	1.000 ± 0.025	1.000 ± 0.037	−0.100	0.925
Day 3	1.088 ± 0.026	0.053 ± 0.060*‡	7.941	0.015
Day 7	0.101 ± 0.007*†	0.007 ± 0.001*†‡	24.012	0.002
Day 14	0.162 ± 0.009*†	0.027 ± 0.005*‡	21.788	0.001
F	63.048	1.903	–	–
P	0.000	0.000	–	–

Values are given as the mean ± SD. *The significance of differences between culture time point and fresh (day 0); †The significance of differences between culture time point and day 3; ‡The significance of differences between static load and controls. *Agg*: Aggrecan; PCR: Polymerase chain reaction; SD: Standard deviation, –: Not applicable.

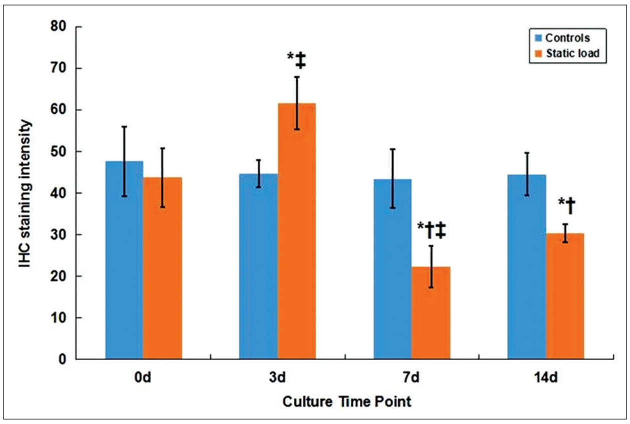

Figure 4: Collagen Type II alpha 1 IHC staining intensity of the NP at each time point. No significant differences in collagen Type II alpha 1 staining intensity were observed between the fresh tissues and controls. The samples under static load showed a significant enhancement at 3 day, and significant differences were observed between the controls and fresh tissues. At 7 day, the staining was obviously decreased, and by 14 days, it was significantly decreased ($P < 0.05$) and significantly different from that of the controls. The values represent the mean ± SD. *$P < 0.05$ versus day 0 of the same group; †$P < 0.05$ versus day 3 of the same group; ‡$P < 0.05$ versus controls. IHC: Immunohistochemistry; NP: Nucleus pulposus; SD: Standard deviation.

to establish conditions for the future study of the effect of complex dynamic loading on IVDs. Abnormal mechanical loading plays an important role in the pathogenesis of IVD degeneration.[4,32-34] Moreover, static loading can lead to alterations in the histological and biochemical properties of the IVDs.[16,35,36] However, the IVDs are the key load-bearing components of the spine, and they are responsible for buffering shock, maintaining spinal stability, and distributing external force.[37] Therefore, it is essential to study the biology and mechanobiology of the IVDs to improve the treatment of disc degeneration and LBP.

A suitable model can provide an experimental platform for related research. All models have advantages and shortcomings depending on the specific hypotheses. Because of the lack of close control and monitoring of the mechanical environment of *in vivo* models, it is difficult to examine specific aspects of cell signal transduction and matrix metabolism.[1] The *in vitro* culturing of isolated disc cells is widely performed to analyze specific factors; however, because they are separated from the original ECM, it is difficult to maintain the original cell properties and interactions between cells, leading to the loss of cell phenotypes.[38] Compared with the above-mentioned methods, culturing of the whole disc organ can enable the maintenance of cell-to-cell interactions, as well as interactions between cells and the ECM, which is conducive to studying the metabolism and degeneration of the discs under controlled conditions. Moreover, this method is convenient for observing the responses of the IVD tissues to specific external stimuli.[39-41]

Accordingly, Lim *et al*.[27] developed an *in vitro* organ culture model of the rat spinal motion segment and demonstrated the maintenance of viability and tissue integrity for 14 days. This spinal motion segment included the VBs-IVDs. In previous studies,[11,15,42] the loading was applied directly to the surface of the IVD organ, and thus, the pressure was applied through the cartilage EP or the AF, leading to a specimen loading environment that significantly differed from the actual biological situation. *In vivo* loading is applied to the bone EP through the VBs, and then the cartilage EP is compressed, and the force is transferred to each part of the AF to avoid uneven stress. Finally, it is transferred to the NP.[43,44] The major difference between our study and the previous studies is that we tested the motion segment, which we believe enabled this *ex vivo* IVD organ model to more closely resemble the actual physiological state and, thus, provided a more accurate simulation of spinal loading conditions. In addition, the adjoining VBs (as rigid fixtures) might facilitate the application and control of complex mechanical loads and, thus, allow for the further study of IVD biomechanics.

Seol *et al*.[28] compared rabbit and rat IVD motion segments and found that glycosaminoglycans (GAGs) loss was minimal in rabbit IVDs but that it was progressive and severe in rat IVDs. Further, the rat IVDs showed increased expression of matrix metalloproteinase-3 and markedly decreased expression of collagen Types I and II compared to the rabbit IVDs. Therefore, the rabbit IVDs were more

Table 4: *COL2A1* gene expression quantified by real-time PCR during organ culture

Culture time point	Controls	Static load	*t*	*P*
Day 0	1.000 ± 0.041	1.000 ± 0.038	0.933	0.404
Day 3	0.506 ± 0.042*	1.195 ± 0.040*,‡	−20.365	0.000
Day 7	0.368 ± 0.058*	0.016 ± 0.002*,†,‡	3.946	0.017
Day 14	0.523 ± 0.028*	0.005 ± 0.001*,†,‡	10.493	0.000
F	14.020	1.561	–	–
P	0.000	0.000	–	–

Values are given as the mean ± SD. *The significance of differences between culture time point and fresh (day 0); †The significance of differences between culture time point and day 3; ‡The significance of differences between static load and controls. *COL2A1*: Collagen Type II alpha 1; PCR: Polymerase chain reaction; SD: Standard deviation; –: Not applicable.

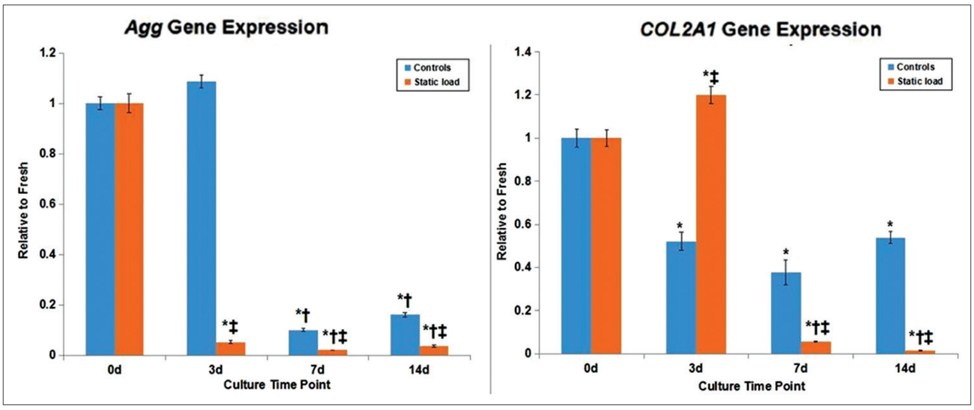

Figure 5: Relative expression quantified by real-time PCR. An obvious downregulation of *Agg* was observed in the controls after 3 days whereas a significant downregulation was observed under static load during culture compared with fresh tissue and controls. An upregulation of *COL2A1* was observed in response to static load by 3 days, and the expression of this gene subsequently decreased until day 14 to below detectable levels. *COL2A1* was downregulated in the controls by 3 days whereas no significant downregulation was observed after culturing, and significant differences were observed between the two groups after culture. The values represent the means ± SD normalized to fresh tissue. *$P < 0.05$ versus day 0 of the same group; †$P < 0.05$ versus day 3 of the same group; ‡$P < 0.05$ versus controls. PCR: Polymerase chain reaction; *Agg*: Aggrecan; *COL2A1*: Collagen Type II alpha 1; SD: Standard deviation.

stable in *ex vivo* culture. Accordingly, we used this motion segment as a model in the current mechanobiological study. Although a decrease in *COL2A1* expression in the nucleus was observed in the control tissues cultured for 3 days compared with the fresh tissues, its expression was stable by 14 days, and the tissue structure was maintained during culturing. Seol *et al*.[28] did not detect any obvious change in the GAG content in motion segments cultured for 14 days; however, in our study, a significant decrease in proteoglycan gene expression was detected in the controls. This result likely occurred because of limited nutrition and the lack of mechanical loading. Regardless, we administered anticlotting agents before donor death and after tissue harvesting to prevent the blood clot formation as previously described.[1,19,45] During prolonged culturing, VBs likely compromise the processing of nutrients through the EP channels,[46] resulting in decreased biosynthetic activity. In addition, removal of the mechanical load significantly affected disc cell production by the matrix,[21,47] and maintenance of the matrix content, as well as cell viability, was expected under mechanical load. Therefore, we will take advantage of this model in future studies.

Numerous *ex vivo* studies[9,41,48] have been performed using modified culture systems that have aimed to elucidate the effects of IVD biomechanics on the maintenance of tissue structure and function. For example, Ohshima *et al*.[15] developed a perfusion apparatus to use osmotic stress *in lieu* of mechanical stress to apply the load experienced by the discs *in vivo*; in addition, Lee *et al*.[19] designed a chamber based on that of Ohshima for long-term culturing to explore the possibility of the *ex vivo* culturing of large disc explants. However, the results revealed a marked decrease in cell viability after one week in the disc EPs. This shortcoming might have been due to the sizes of the discs, given that the organ culturing of large animal IVDs requires systemic anticoagulation.[49] Therefore, Gantenbein *et al*.[1] used anticoagulation treatments to avoid blood clotting in the EP capillary beds and established a bioreactor to apply diurnal loading to an ovine caudal IVD for 7 days; Jünger *et al*.[49] improved this culture system by applying low-magnitude cyclic loading in addition to diurnal loading. Further, Chan *et al*.[23] used a dynamic loading bioreactor to study the effect of more complex loading on disc degeneration. Although the previous devices were applicable for analysis of the IVD organ, they were not suitable for the culturing or loading of the IVD motion segment because the middle disc is softer than both sides of the VBs; moreover, the vertical axis was longer than the IVD organ. The segment was prone to bending deformation under loading, leading to difficult application of vertical compression and easily damaged disc tissues. Therefore, we designed a loading and organ culturing apparatus for the IVD motion segment. As described previously, the use of jackscrews with pedestals enabled maintenance of the motion segment in an upright position, and the mobile loading plate ensured that full vertical loading was consistently applied on the surface of the model [Supplementary Figure 3].

In this experiment, the osmolarity of the medium was adjusted to 410 mOsm/kg to control disc swelling.[17] A pilot experiment estimated that the weight can produce a disc compression force of 0.55 ± 0.12 MPa. Studies have shown that the IVDs are subjected to pressure ranging from 0.1 to 1.1 MPa during the day[23,50] and that compressive loading of over 0.8 MPa could induce early disc degeneration.[9,51] Because data on physiological loading magnitudes are incomplete for rabbit IVDs, the loading magnitudes applied in this study were based on the physiological range of loading in humans.

Over the 14 days of culturing under constant static compression, the morphological integrity of the IVDs gradually deteriorated after 3 days, and the proteoglycan content of the NP was significantly decreased compared with that in the control tissues cultured in 6-well plates. However, the collagen Type II alpha 1 content was obviously increased at 3 days in the control tissues compared with the fresh tissues, after which it was markedly reduced. qRT-PCR analysis also confirmed that *Agg* gene expression was significantly downregulated and significantly different than that in the controls; however, *COL2A1* gene expression was upregulated at 3 days, and this trend was consistent with the results of histological and IHC analyses. These phenomena might be explained by Wolff's law,[52] which states that an appropriate mechanical load can allow for the maintenance of tissue morphology and can stimulate bone metabolism to synthesize matrix components; however, a persistent or excessive load inhibits these functions. Ohshima *et al*.[15] assessed the effect of static loading on intact bovine coccygeal discs and found that maximum tissue hydration occurred at a load of 5–10 kg and that lighter (0.5 kg) or heavier loads (15 kg) led to decreased incorporation. In the current experiment, in the presence of intradiscal pressure produced by a load of 3 kg, IHC and RT-PCR analyses revealed that the synthesis of collagen Type II alpha 1 was stimulated to resist compression within a brief period (3 days); hence, the collagen Type II alpha 1 content was higher compared with those in the no-loading controls and fresh tissues. However, the constant static compression inhibited cell metabolic activity and led to a significant decrease in the collagen content after brief stimulation.

During culture, however, the proteoglycan content did not increase. This finding might have been observed because the static load significantly inhibited the production of proteoglycans. Lee *et al*.[19] cultured bovine coccygeal discs under a 5 kg static load and found that although NP cell viability was maintained after 1 week, an approximately 50% decrease in proteoglycan synthesis occurred within 2 days of culturing. In contrast, Gantenbein *et al*.[1] cultured models under cyclic diurnal loading (0.2 MPa for 6 h and 0.8 MPa for 16 h) and demonstrated that GAG synthesis was maintained after 7 days, showing that frequent loading can stimulate and maintain proteoglycan synthesis. Under general conditions, the changes in the levels of *COL2A1* and *Agg* are consistent;[10-13,16-22] however, Neidlinger-Wilke *et al*.[53] found that IVDs under low hydrostatic pressure (0.25 MPa, 30 min, 0.1 Hz) tend to exhibit increased *Agg* expression in cell nuclei and decreased *COL2A1* expression. Whereas the high hydrostatic pressure tended to decrease the expression of both *Agg* and *COL2A1*, the author suggested that hydrostatic pressure might regulate disc matrix turnover in a dose-dependent manner. Our experiment further showed that the changes in the two main matrix molecules were inconsistent up to 3 days and that the proteoglycan content was significantly decreased after 3 days but that collagen Type II alpha 1 production was briefly stimulated. The differences between our results and those of the above-mentioned studies are mainly attributed to differences in loading styles; a constant static load can significantly inhibit the production of proteoglycans.[17,19,24,54] In addition, static loading might have stronger effects on proteoglycans than on collagen Type II alpha 1. Thus, a shorter observational period will be used in a future study to test this hypothesis.

Future studies should aim to improve the *ex vivo* culture system and apparatus for application of a consistent physiologic load and complex dynamic load to maintain the functions of the model over a longer duration. In addition, the *ex vivo* model should be used to further examine IVD biomechanics and to observe the means through which degenerative discs can be manipulated to provide a basis for the clinical treatment of degenerative disc diseases.

In conclusion, a loading and organ culture system for *ex vivo* rabbit IVD motion segments was developed. Although degeneration was observed in the fresh IVDs under the no-loading conditions, the tissue architecture and some of the matrix synthesis activity were maintained in the motion segment culture. However, constant static compression led to significant IVD degeneration, and specifically to a change in the proteoglycan content, which was markedly decreased after culturing. Nevertheless, we found that static compression stimulated the synthesis of collagen Type II alpha 1 within a brief time period. Thus, the results of this study indicate that an appropriate mechanical load can stimulate matrix synthesis but that constant static compression leads to progressive disc degeneration.

Supplementary information is linked to the online version of the paper on the Chinese Medical Journal website.

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Conflicts of interest

There are no conflicts of interest.

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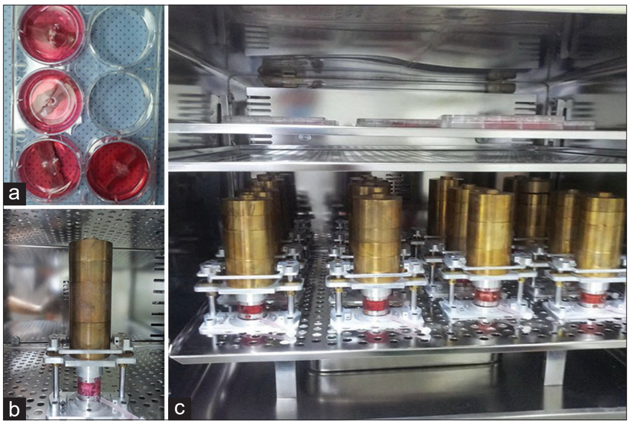
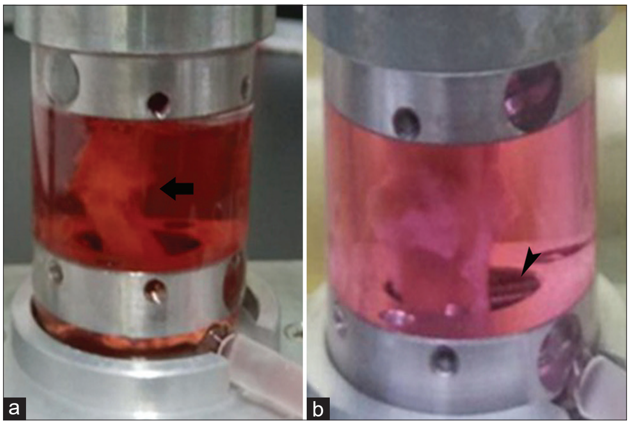
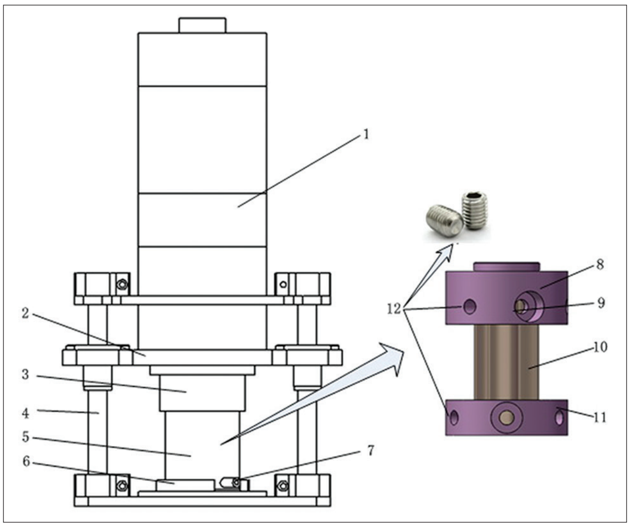


Supplementary Figure 1: Detailed view of the specimen culturing conditions. (a) The specimens were maintained in no-loading 6-well plates for the control conditions. (b) The specimens were maintained in load apparatuses under a constant compressive load. (c) The 6-well plates and apparatuses were situated inside of a 37°C incubator with 5% CO_2 and 100% humidity.



Supplementary Figure 3: Effect of fixation though jackscrews with pedestals. IVD motion segments are prone to bending (arrow) under loading without fixation (a). The specimen can be maintained in an upright state through fixation with jackscrews (arrowhead) though the pedestals (b) to ensure that full vertical loading is consistently applied on the surface of the model. IVD: Intervertebral disc.



Supplementary Figure 2: Loading and organ culturing apparatuses for the rabbit IVD motion segments (diagrammatic sketch). 1: Weights, 2: Loading plate, 3: Gland, 4: Optical axis, 5: Chamber, 6: Chamber fixed base, 7: Outlet, 8: Top pedestal, 9: Fluid level observation hole, 10: IVD motion segments, 11: Base pedestal, and 12: Jackscrews. IVD: Intervertebral disc.