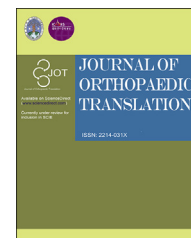


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## ORIGINAL ARTICLE

# Development of a whole organ culture model for intervertebral disc disease



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**Summary** *Background/Objective:* Whole organ *in vitro* intervertebral disc models have been associated with poor maintenance of cell viability. No previous studies have used a rotating wall vessel bioreactor for intervertebral disc explants culture. The purpose of this study was to develop and validate an *in vitro* model for the assessment of biological and biomechanical measures of intervertebral disc health and disease.

*Methods:* To this end, endplate-intervertebral disc-endplate whole organ explants were harvested from the tails of rats. For the injured group, the annulus fibrosus was penetrated with a 20G needle to the nucleus pulposus and aspirated. Explants were cultured in a rotating wall vessel bioreactor for 14 days.

*Results:* Cell viability and histologic assessments were performed at Day 0, Day 1, Day 7, and Day 14. Compressive mechanical properties of the intervertebral disc were assessed at Day 0 and Day 14. In the annulus fibrosus and nucleus pulposus cells, the uninjured group maintained high viability through 14 days of culture, whereas cell viability in annulus fibrosus and nucleus pulposus of the injured intervertebral discs was markedly lower at Day 7 and Day 14. Histologically, the uninjured intervertebral discs maintained cell viability and tissue morphology and architecture through 14 days, whereas the injured intervertebral discs showed areas of cell death, loss of extracellular matrix integrity, and architecture by Day 14. Stiffness values for uninjured intervertebral discs were similar at Day 0 and Day 14, whereas the stiffness for the injured intervertebral discs was approximately 2.5 times greater at Day 14.

*Conclusion:* These results suggest that whole organ intervertebral discs explants can be successfully cultured in a rotating wall vessel bioreactor to maintain cell viability and tissue architecture in both annulus fibrosus and nucleus pulposus for at least 14 days. In addition, the

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injury used produced pathologic changes consistent with those seen in degenerative intervertebral disc disease in humans. This model will permit further study into potential future treatments and other mechanisms of addressing intervertebral disc disease.

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## Introduction

Intervertebral disc (IVD) disorders (desiccation, degeneration, herniation, etc.) have been directly associated with low back pain and disability [1]. Current treatment options for IVD disorders do not restore normal tissue integrity or function. The direct medical and indirect costs of these conditions are unknown, but have been estimated in the range of \$50 to \$100 billion per annum, placing an economic burden on society [2–4]. Thus, the development of prevention strategies for disc degeneration and repair/regeneration options for pathologic tissues would seem to be of great value. Unfortunately, mechanisms of disc degeneration are not fully understood, although aging, injury, genetics, nutrition, metabolism, and mechanical stress are all suspected to be significant factors [4–6].

*In vitro* culture models provide a controlled method for investigating mechanisms of disc degeneration and can be performed using cells, single tissues, or whole organs. Models using cells alone allow for the control of certain variables and are typically less complex and expensive to employ than other options [5]. However, monolayer cell culture models deprived of extracellular matrix (ECM) commonly result in rapid cell dedifferentiation and/or loss of cell viability [7]. In addition, these cell-alone culture models call into question their validity for assessment of clinically relevant outcome measures such as biomechanics and morphological integrity. Tissue cultures of IVDs without the adjacent endplates allow for better maintenance of cell distribution and differentiation, ECM integrity, and material properties, but the biologic and biomechanical influences of endplate cartilage and vertebral bone are lost, and the nucleus pulposus (NP) is allowed to freely swell in culture [8,9]. Based on these limitations, establishing a valid whole organ culture model of IVD is desirable. Ideally, this model would provide long-term maintenance of cell and tissue integrity, architecture, composition, and cell viability; allowing for the assessment of biological and biomechanical aspects of disc physiology and pathology.

In the last decade, there has been growing interest in using organ culture models to investigate the effects of injury, degeneration, or repair [10,11]. Once validated, these *ex vivo* models could provide an excellent method for evaluating disease mechanisms and therapeutic strategies regarding the disc's biological and mechanical functions in a controlled culture environment. Early studies demonstrated that culturing the intervertebral disc *ex vivo* with the vertebral bodies attached resulted in a decrease in the amount and distribution of living cells due to limited nutrient diffusion [5,7,9]. Some models employ the removal of the endplates to maintain cell viability [7,12]; however, the endplate is crucial for constraining the NP, which has

extreme swelling capabilities, and for maintaining the *in situ* nutrient diffusion pathways [13]. Additionally, many of these studies have been limited to histological and gene expression analyses, excluding the critical mechanical functions of the disc. Therefore, it is important for a validated organ culture model to maintain cell viability, biochemical, and mechanical properties.

Translational research using animal models for eventual clinical application of *in vitro* studies will be a necessary step for this work. Unfortunately, there is no ideal animal model for the study of human disc degeneration [14]. Small animal models are beneficial for studying genetic alterations and providing a cost effective option for mechanistic research. Large animal models allow for more clinically relevant interventions and outcomes measures, but are more costly and labour intensive. Previous animal model studies using needle puncture to damage the disc have been successful in causing reproducible degenerative-like changes over time, including a decrease in disc height, water content, and glycosaminoglycan content [15]. Developing an *in vitro* model based on these successful *in vivo* degeneration models would provide an efficient, cost-effective method for initial translational research prior to performing experiments requiring the use of animals.

The aims of this study were to: (1) develop a whole organ IVD culture model that would allow for maintenance of cell viability, ECM integrity, and mechanical function for a relevant time period; and (2) produce degenerative-like changes in the disc *in vitro*. To the authors' knowledge, there have been no reports on whole organ IVD culture using a rotating wall vessel (RWV) bioreactor. This study employed the use of a RWV bioreactor as a novel approach for development and initial validation of a whole – organ IVD culture system, with or without nucleotomy, for the study of biological and biomechanical aspects of disc health and disease. We hypothesized that: (1) cell viability, ECM integrity, and compressive material properties of discs could be maintained for 14 days in whole organ IVD culture using a RWV bioreactor at levels not significantly different than at time of harvest; and (2) that needle-puncture nucleotomy and NP aspiration would be associated with significant loss of cell viability, ECM composition and architecture, and compressive material properties over 14 days in whole organ IVD culture using a RWV bioreactor.

## Materials and methods

### Preparation of IVD explants

Under Animal Care and Use Committee approval, tails were collected from 12 skeletally mature Sprague-Dawley rats after they were euthanised for reasons unrelated to this study. The muscles and tendons were dissected and

removed carefully from the caudal vertebrae using a scalpel and rongeurs under aseptic conditions. Explants consisting of the cranial body half, cartilage endplate, IVD, cartilage endplate, and caudal body half were then harvested using a saw (Figure 1). The IVD whole organ explants were randomly assigned to one of two groups: injured or uninjured. For the injured group, the posterolateral annulus fibrosus (AF) of each explant was penetrated with a 20G needle to enter the approximate centre of the NP based on depth of needle penetration and tactile feel. Aspiration of the NP was then performed using a 1 mL syringe with the plunger pulled to 0.5 mL in order to model whole-disc pathology, including both AF and NP insults, as is typically seen in symptomatic IVD disease in patients ( $n = 20$ ; Figure 1) [14]. The uninjured group received no insult ( $n = 22$ ; Figure 1).

### Whole organ culture of IVD

For both groups, the explants were cultured in a RWV bioreactor (RCCS-45C, Synthecon Inc., Houston, TX, USA) at 50 rpm for 14 days in an incubator at 5% CO<sub>2</sub> and 37 °C (Figure 1). Explants were free floating in the system as seen in Figure 1. Explants were cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 mg/mL ascorbic acid, 1 × MEM N-E amino acid solution, 1% insulin transferrin selenium (ITS premix: BD Biosciences, Bedford, MA, USA), and 1 × penicillin-streptomycin (all components are from Invitrogen Co., Carlsbad, CA, USA unless otherwise specified). Culture media were changed at Day 1 and Day 7 of culture.

### Cell viability

Samples designated for cell viability and histologic assessments ( $n = 5$  at Day 0, Day 1, Day 7, and Day 14 for the injured group.  $N = 6$  at Day 0 and Day 14, and  $n = 5$  at Day 1 and Day 7 for the uninjured group) were prepared first for cell viability assay. Cell viability in each IVD was examined using fluorescent live/dead assay immediately after preparation (Day 0), and at Day 1, Day 7, and Day 14 of culture. The explants were cut along the mid-disc height with a scalpel blade. Then the explants were rinsed with Dulbecco's phosphate-buffered saline (Invitrogen) and incubated

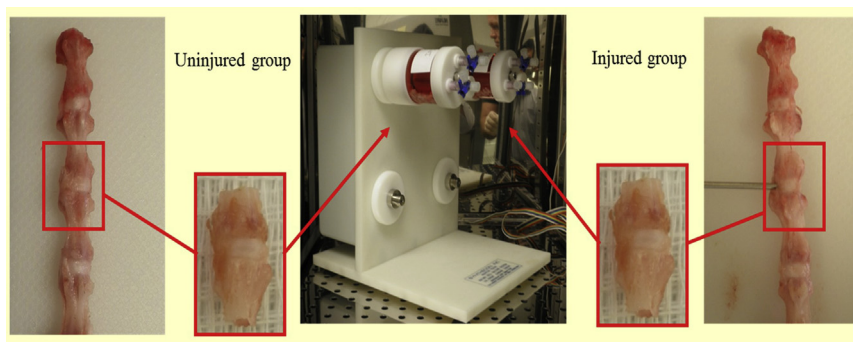
in Dulbecco's phosphate-buffered saline with 4 μL/mL calcein-AM (Invitrogen), and 1 μL/mL ethidium homodimer-1 (Invitrogen) for 25 minutes at room temperature, protected from light. The discs were visualised with a fluorescent microscope (BX-51; Olympus Co., Tokyo, Japan). Using these stains, live cells were stained green by calcein-AM, and dead cells stained red by ethidium homodimer-1. Objective cell viability analysis for the AF was conducted using custom in-house software. Viable cell counts were divided by the measured area in order to control for different sizes in explants.

### Histology

IVD tissues were fixed in 10% neutral buffered formalin after cell viability testing. These tissues were dehydrated with gradually increasing concentrations of ethanol and embedded in paraffin. Coronal sections of IVD tissues were cut to 6 μm and mounted onto microscope slides. The samples were then dewaxed, rehydrated, and stained with haematoxylin eosin to evaluate the tissue structure and cell morphology. In addition, the sections were stained with Safranin-O (477-73-6; Sigma-Aldrich, St. Louis, MO, USA) and Masson's trichrome (KTMTR; American MasterTech, Lodi, CA, USA) to determine the distribution and quantity of proteoglycan and collagen matrix, respectively. These samples were subjectively assessed for cell and tissue morphology and architecture by one pathologist who was blinded to the sample information.

### Biomechanical testing

Samples designated for biomechanical testing ( $n = 5$  at Day 0 and Day 14 for the injured group and  $n = 6$  at Day 0 and Day 14 for the uninjured group) were frozen and shipped to Columbia University overnight and stored at -20°C until mechanical testing was performed. The top and bottom surfaces of semi-frozen bone-disc-bone motion segments were made parallel for mechanical testing using an electronic sander (Delta ShopMaster Type I; DELTA Power Equipment Co. Anderson, SC, USA). Then, samples were completely thawed and allowed to rehydrate for 1 hour. Unconfined compression was performed on a custom device at a quasistatic loading rate (0.001 mm/s) at Day 0 and Day 14. Since initial radiographs were not available to measure



**Figure 1** Depicts the creation of both injured and uninjured whole organ explant including cranial body half, cartilage endplate, intervertebral disc, cartilage endplate, and caudal body half.

the initial disc height, the initial disc height was assumed to be 0.94 mm for the rat caudal disc [14]. Force and displacement values were recorded and the stiffness of the disc was calculated as the slope of the toe- and linear-region of the force-displacement curve. To determine the effect of *in vitro* culture on the mechanical properties of the joint, stiffness measured at Day 0 was compared to the stiffness measured at Day 14 using Student *t* test. To determine the effect of injury on the joint mechanical properties, the stiffness for discs in the uninjured group was compared to the injured group using Student *t* test.

## Results

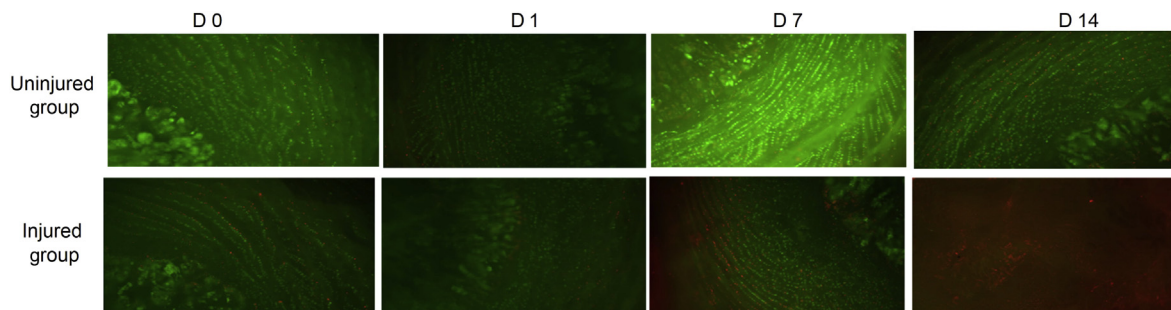
### Cell viability

Based on subjective assessment, AF and NP cells in the uninjured group maintained >90% viability in all sections evaluated through 14 days of culture. The viability of AF and NP cells was similarly high in the injured group at Day 1 of culture. In contrast, cell viability at the injury site in the AF and within the NP of the injured IVDs was markedly lower throughout the IVD (<50%) at Day 7 and Day 14 of culture (Figure 2).

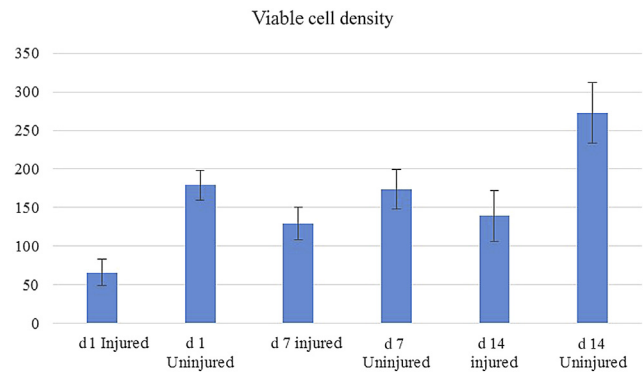
AF viable cell counts were significantly ( $p < 0.05$ ) higher in the uninjured group as compared with the injured group at Day 1 and Day 14. Interestingly, AF viable cell counts increased from Day 1 to Day 14 in the injured group although this difference was not statistically ( $p = 0.1$ ) significant. AF viable cell counts increased significantly ( $p < 0.05$ ) from Day 1 to Day 14 in the uninjured group (Figure 3).

### Histology

The uninjured IVDs maintained normal cell and tissue morphology and architecture through 14 days of culture. The injured IVDs showed areas of cell death characterised by pyknotic nuclei corresponding to the loss of cell viability noted, as well as a loss of collagen and ECM integrity and architecture by Day 14 of culture (Figure 4). The NP showed a more intense orange-red colour, consistent with higher levels of proteoglycan staining at Day 1 of culture in both the injured group and uninjured group as compared with Day 7 and Day 14 (Figures 4 and 5). At Day



**Figure 2** Subjective cell viability images stained with calcein and ethidium homodimer at Day 0, Day 1, Day 7, and Day 14. Images reveal that both groups had >90% viability on Day 0. Furthermore, the uninjured group maintained >90% viability through 14 days of culture while the injured group contained markedly lower viability.

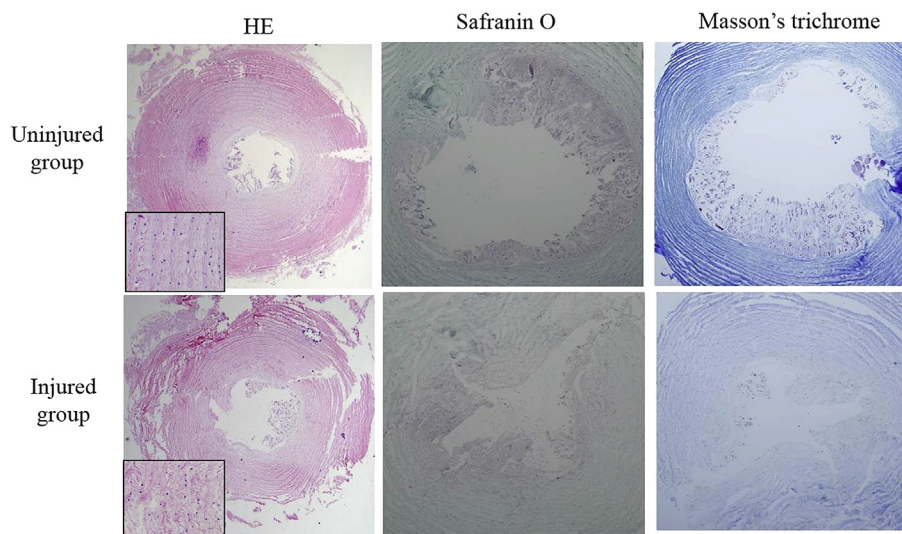


**Figure 3** Viable cell density was significantly higher ( $p < 0.05$ ) in the uninjured group as compared to the injured group at Day 1 and Day 14. Annulus fibrosus viable cell counts increased in both groups although only significantly in the uninjured ( $p < 0.05$ ) group.

14 of culture, the uninjured group showed a more intense proteoglycan staining as compared with the injured group (Figure 4). Based on Masson's trichrome staining, the AF maintained blue stained collagen in orderly arranged circumferential fibres through Day 14 of culture in the uninjured group. In the injured group, the AF architecture became distorted and the Day 14 collagen staining in the AF was less intense than at Day 1. The collagen staining intensity in the AF at Day 14 of culture in the injured group was less than in the uninjured group (Figure 4). These findings suggested that cultured IVDs in the uninjured group maintained near-normal cell and ECM morphology and composition were well preserved in cultured IVDs in uninjured group for at least 14 days, while injured discs underwent degenerative-like changes not seen in the uninjured group.

### Biomechanical testing

Stiffness was calculated as the slope of the force-displacement curve in the toe- and linear-regions. For the uninjured group, the toe- and linear-region stiffness measured at Day 14 was not significantly altered from the initial properties measured at Day 0 (Figure 6A;  $p > 0.3$ ). At Day 0, the toe-region stiffness of the uninjured group was

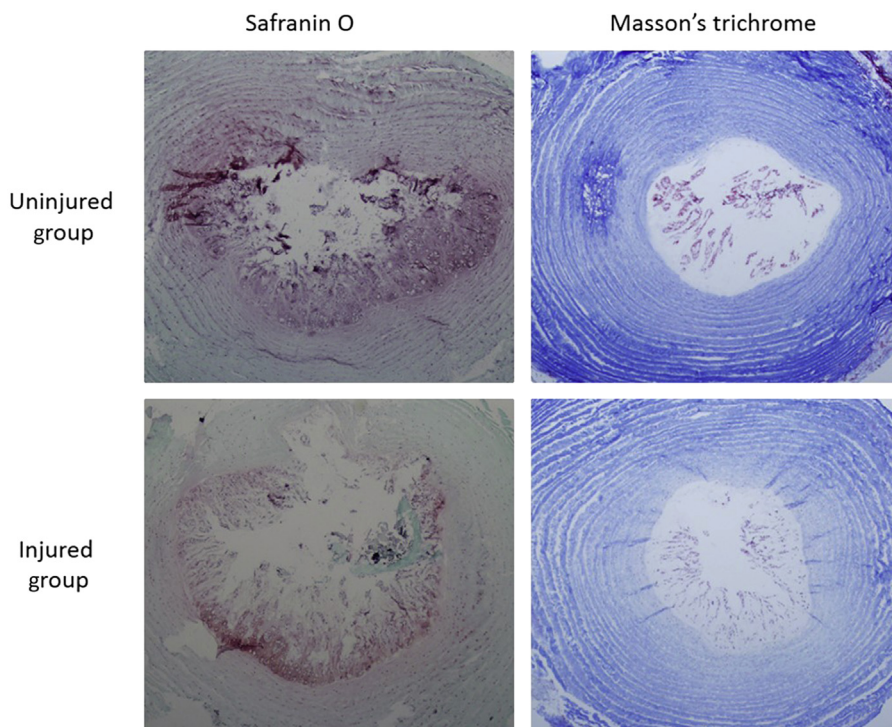


**Figure 4** Histology of intervertebral disc (IVD) tissues at Day 14 of culture. The uninjured IVDs maintained normal cell and tissue morphology and architecture through 14 days of culture, whereas injured IVDs showed areas of cell death characterised by pyknotic nuclei (inset in injured group HE stain) corresponding to the loss of cell viability noted, as well as a loss of collagen, extracellular matrix integrity, and distorted architecture by day 14 of culture. HE = haematoxylin and eosin stain.

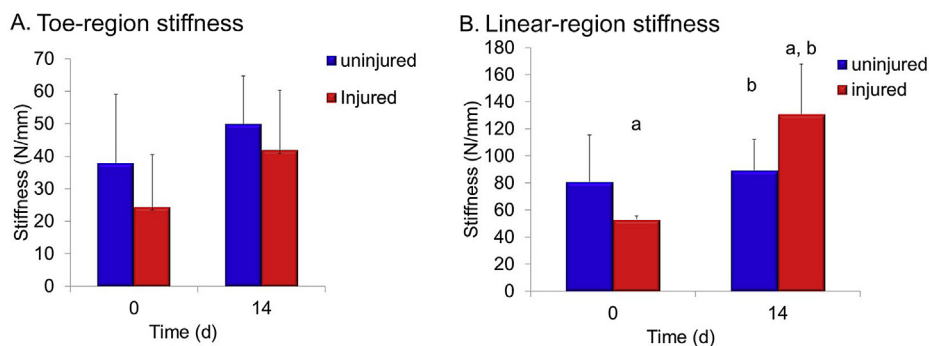
$37.9 \pm 21.2$  N/mm and the linear-region stiffness was  $80.8 \pm 34.8$  N/mm. For the injured group, the linear-region stiffness was approximately 2.5 times greater at Day 14 compared to Day 0 ( $p < 0.01$ ; Figure 6B). The toe-region stiffness of the injured group was not significantly altered with culture time ( $p = 0.1$ ).

The applied injury to the NP through the AF did not initially alter the mechanical function; however, with time

in culture, differences were observed between the injured and uninjured discs. The applied injury did not significantly alter the toe- or linear-region stiffness measured at Day 0 ( $p > 0.1$ ). However, at Day 14, the linear-region stiffness for the injured group was 50% higher than the uninjured group ( $p = 0.04$ ; Figure 6B). There was no significant difference in the toe-region stiffness between the injured and uninjured groups at Day 14 ( $p = 0.4$ ).



**Figure 5** Histology of intervertebral disc tissues at Day 0 of culture. Both groups of intervertebral discs contained normal cell and tissue morphology and architecture at Day 0.



**Figure 6** Tissue stiffness calculated using the slope of the force-displacement curve in the toe and linear regions.

## Discussion

The results of this study suggest that whole-organ rat IVD explants can be successfully cultured in a RWV bioreactor to maintain cell viability, tissue composition, and architecture, and mechanical properties for at least 14 days. In addition, we were able to perform multiple clinically relevant outcome measures on IVD explants to assess biologic and biomechanical aspects of IVD physiology and pathology. These data represent a significant advance in development towards a valid *in vitro* model for translational study of IVD health and disease.

Despite progress in methods for organ culture in recent years, it has proven difficult to maintain cell viability for extended periods of time when culturing IVD with cartilage endplates and adjacent vertebral bone attached. In previous studies, cell viability and density of IVDs in whole organ culture models significantly decreased during the culture period, likely as a result of the avascular nature of the IVD in combination with large diffusion distances limiting the transport of nutrients into and waste out of the IVD [6]. Lee et al [16] reported a remarkable drop in cell viability to nearly 0% in the NP of bovine caudal discs cultured with cartilage endplates for 7 days. When the cartilage endplates were removed, there was no apparent loss of viability over the 7 days in culture [16]. Risbud et al [17] documented that cell viability in rat lumbar disc explants cultured with cartilage endplates using static hyperosmotic culture media was reduced by 25% after 7 days. Gawri et al [9] reported that culturing human IVD with cartilage endplates plus adjacent vertebral bone for 7 days resulted in decreases in cell viability of 96% and 86% in the NP and AF, respectively. The results of these studies suggest that insufficient nutrient supply leading to cell death in IVDs cultured for 7 days or more has been a major limitation for developing an optimal *in vitro* model for study of disc disease.

Mechanical stress is another critical factor involved in IVD health and disease [18–21], and the disc experiences complex loading conditions *in vivo* that are difficult to accurately replicate *in vitro* [20–22]. Recently, investigators have used various bioreactors in an attempt to mimic diurnal gravitational loading in IVD organ culture models to improve cell viability and provide mechanical stress to tissues [16–18]. Gantenbein et al [5] reported that cell viability remained unchanged over 7 days when whole

ovine caudal IVDs with cartilage endplates were cultured in a bioreactor under continuous uniaxial diurnal loading. Haglund et al [13] reported cell viability of 88% and 80% in the NP and AF, respectively, when bovine caudal discs with cartilage endplates were cultured using an axially loading bioreactor for a total of 4 weeks. The authors of those studies promote the use of bioreactors to instigate nutrient flow across the endplate and preserve cell and tissue viability [23–25]. RWV bioreactors provide a microgravity environment for cell culture [25] fluid-flow shear stresses on cultured tissues [23–26]. The shear stresses generated by the laminar flow of a rotating vessel along a horizontal axis appear have been reported to be effective in reducing diffusional limitations of nutrients, oxygen, and waste products [23] and providing a method for mechanical signalling and communication among cells [24]. RWV bioreactors have been demonstrated to be effective in these capacities using engineered bone [26–28] and cartilage [28–32], and to facilitate engineering of tissues and organs for *in vitro* model systems of tissue development and function [23–25]. Therefore, the present study used a RWV bioreactor for rat IVD whole organ culture in an attempt to improve cell nutrition and viability in the AF and NP of IVDs with attached endplates and vertebral bone, and provide some degree of mechanical stress on IVDs for the 14-day culture period. This methodology proved successful in that cell viability, tissue composition, and architecture, and material properties in both the AF and NP of uninjured discs could be maintained for at least 14 days using a RWV bioreactor. These data suggest that media fluid flow is a vital factor in maintaining cell viability and tissue integrity for extended periods in whole organ IVD cultures.

Annular injury has been used to induce stimulated degeneration in various *in vivo* animal models in order to investigate the aetiology and mechanisms of disc degeneration [33]. Needle puncture, concentric tear, radial tear, transverse tear at mid-plane, and transverse tear at the endplate rim have been reported as annular injury methods [33]. Among these methods, the simplest method to induce disc degeneration is a needle puncture through the annulus [33,34]. Percutaneous annulus needle puncture in rat tail discs results in decreased NP volume, annular layer disorganisation, inward bulging of the inner annulus, and annular tears, which are similar to the degenerative changes reported for human degenerative IVD disorders and animal models previously described [33–35]. Puncture size

is an important determinant of the observed rate of disc degeneration in animal models. It was reported that when the needle diameter was greater than 40% of the disc height, the disc changes were universal [36]. Hsieh et al [33] reported that degenerative changes in the AF were induced by 18G, but not 22G or 26G, percutaneous needle punctures in rat tail discs. In the present study, injury with a 20G needle with aspiration of NP produced pathologic changes in the AF and NP similar to those seen in degenerative IVD disease in animal models and human clinical patients, including cell death, abnormal ECM, and increased stiffness. In previous studies, needle puncture has been reported to directly alter IVD mechanical properties via AF damage and NP depressurisation [36], and that puncture injury reduces disc stiffness [37]. Iatridis et al [38] demonstrated that puncture using a relatively large-size needle (>40% of disc height) decreases the disc axial stiffness, and loss of stiffness is exhibited only when an injury fully penetrates the AF. Another report documented that 25G to 30G needles did not affect disc compressive mechanics in a rat tail model [36]. In the present study, stiffness values of the discs measured immediately after injury were lower than those of the healthy disc due to the compromised nature of the AF. However, these initial differences in stiffness were not statistically significant, and at Day 14, the linear-region stiffness for the injured group was approximately 250% greater than at Day 0 and 50% higher than Day 14 values for the uninjured group. This increase in stiffness for the injured discs in the present study was associated with degenerative changes including cell death and loss of ECM integrity and architecture. Similarly, studies using *in vivo* rat tail models also reported degenerative discs to have increased stiffness [38–40]. In humans, age-related disc degeneration causes increased stiffness [41,42]. It has been reported that an increase in the stiffness of the disc is caused by decreases in water content, number of fibres in the NP, and AF thickness [40–43]. For the present study, the AF was penetrated with a 20G needle and the NP was aspirated as an aggressive *in vitro* model of IVD disease aimed at seeing clinically relevant pathologic changes in a 14-day culture period. The data suggest that this aggressive *in vitro* model mimics biomechanical changes associated with disc degeneration in human patients and *in vivo* animal models.

There are limitations to the current study. For this initial study aimed at developing a whole organ IVD culture model for translational research, we used rat tail IVDs cultured in a RWV bioreactor with assessments at 1 day, 7 days, and 14 days. The small size of the rat tail discs entails marked differences in nutrition and biomechanics when compared to large animal and human IVDs, such that direct comparisons regarding maintenance of cell viability and material properties cannot be made [44,45]. In addition, the RWV bioreactor cannot mimic the complex loading patterns and levels that occur *in vivo* [46,47]. However, biomechanical and biologic properties and responses of rat tail IVDs are such that their use for study of mechanisms of disease and screening of interventional strategies are valid [45,48]. Now that we have provided initial evidence for the validity and usefulness of this model, ongoing studies are aimed at the optimisation and expansion of the model to address these limitations and investigate detailed disease

mechanisms, as well as potential preventative and therapeutic strategies for IVD disorders.

## Conclusions

Establishing an *in vitro* whole organ IVD culture model that allows for comprehensive testing prior to use of animal models is highly desirable. The data from the present study suggest that the rat tail whole organ IVD with endplates and vertebral bone model described provides a viable approach to establishing such a model. The results of this study suggest that use of the RWV bioreactor is critical for extended culture of the whole organ IVD explants for maintaining viability, tissue composition and architecture, and material properties. In conclusion, this novel model has high potential for providing important translational data for delineating disease mechanisms and developing therapeutic strategies for IVD disease in humans.

## Conflicts of interest

The authors have no conflicts of interest to declare.

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