Eur Spine J (2011) 20:1244–1254
DOI 10.1007/s00586-011-1721-x

ORIGINAL ARTICLE

Development of an intact intervertebral disc organ culture system in which degeneration can be induced as a prelude to studying repair potential

Bernice Jim · Thomas Steffen · Janet Moir · Peter Roughley · Lisbet Haglund

Received: 27 June 2010 / Revised: 28 December 2010 / Accepted: 6 February 2011 / Published online: 19 February 2011

Abstract The present work describes a novel bovine disc organ culture system with long-term maintenance of cell viability, in which degenerative changes can be induced as a prelude to studying repair. Discs were isolated with three different techniques: without endplates (NEP), with bony endplates (BEP) and with intact cartilage endplates (CEP). Swelling, deformation, and cell viability were evaluated in unloaded cultures. Degeneration was induced by a single trypsin injection into the center of the disc and the effect on cell viability and matrix degradation was followed. Trypsin-treated discs were exposed to TGFβ to evaluate the potential to study repair in this system. NEP isolated discs showed >75% maintained cell viability for up to 10 days but were severely deformed, BEP discs on the other hand maintained morphology but failed to retain cell viability having only 27% viable cells after 10 days. In CEP discs, both cell viability and morphology were maintained for at least 4 weeks where >75% of the cells were still viable. To mimic proteoglycan loss during disc degeneration, a single trypsin injection was administered to the center of the disc. This resulted in 60% loss of aggrecan, after 7 days, without affecting cell viability. When TGFβ was injected to validate that the system can be used to study a repair response following injection of a bio-active substance, proteoglycan synthesis nearly doubled compared to baseline synthesis. Trypsin-treated bovine CEP discs therefore provide a model system for studying repair of the degenerate disc, as morphology, cell viability and responsiveness to bio-active substances were maintained.

Keywords Intervertebral disc · Degeneration · Repair · Organ culture

B. Jim · T. Steffen · J. Moir · L. Haglund (✉)
Orthopaedic Research Laboratory, McGill University,
687 Pine Avenue West, Room L4.70, Montreal,
QC H3A 1A1, Canada
e-mail: lisbet.haglund@mcgill.ca

P. Roughley
Shriners Hospital for Children, McGill University,
1529 Cedar Avenue, Montreal, QC H3G 1AG, Canada

Introduction

One of the major causes of low back pain is degeneration of the intervertebral discs (IVDs). A contributing factor leading to IVD degeneration is the absence of vasculature entering the disc [3, 14], leading to slow exchange of nutrients and waste products across the endplates. The cartilaginous endplate is often calcified with age [41], which might further compromise nutrient flow into the disc. The IVD is only sparsely populated with cells, which are surrounded by a vast extracellular matrix (ECM). Cell number decreases and cell senescence increases with age and degeneration, especially with disc herniation, and the exposure to cytokines [13, 26, 39]. This is also likely a contributing factor to the disc cell's difficulty in maintaining the integrity of the extracellular matrix and the onset of degeneration [37].

The IVD is divided into two distinct zones, the outer fibrous annulus fibrosus (AF) and the central cartilaginous nucleus pulposus (NP) [15]. The AF forms a fibrous outer ring that encloses the more gelatinous NP. The highly anionic proteoglycan aggrecan is entrapped in the collagen fibril network of the NP, and provides the swelling properties important for resistance to compression [48]. Aggrecan fragmentation and loss is one of the hallmarks of disc degeneration and occurs early in the degenerative

process, but also in normal ageing, and has commonly been used as a marker of disc degeneration [5, 21]. Loss of aggrecan can be due to either an increase in proteinase production or a decrease in aggrecan synthesis. The disc also possesses a variety of leucine-rich repeat (LRR) proteins, including chondroadherin (CHAD) [25, 35, 45], which interacts with both the collagen fibrils and the cells, so regulating cell metabolism and ECM structure [4, 25, 29]. CHAD has been associated with premature degeneration in scoliotic discs [2, 11], but there is no published data regarding CHAD and degenerative disc disease. In articular cartilage, CHAD has been shown to be associated with collagen fibers, where it is thought to maintain the integrity of the collagen network [29, 36, 45]. Although currently no information is available on CHAD function in the disc, it is likely to have a similar function to that in articular cartilage. As CHAD may play a role in promoting matrix homeostasis, changes in its abundance or structure might be expected to contribute to pathological changes in the tissue. It is commonly thought that the degradative changes in the ECM are detrimental to disc function, a property that is exacerbated by the accumulation and slow removal of the ECM degradation products. The rate at which these changes occur may vary between individuals, but they are thought to form the basis of the tissue loss associated with disc degeneration.

Numerous animal models have been used to study IVD degeneration. Large animal models using ovine and porcine discs have been accepted as good models for studying disc structure, geometry, biochemistry and biomechanics [34, 49], whereas small animal models using rabbits and rats have in general been used to answer metabolic questions [19]. While the large animals have a disc structure analogous to humans, they undergo degeneration slowly and are expensive to use. In contrast, the small animals are relatively cheap and undergo degenerative changes rapidly, but their disc structure is different to humans, particularly in the maintenance of notochordal cells throughout life [16, 17]. An organ culture model using discs from large animals would be an ideal system to study both disc degeneration and repair. Organ culture models described to date have two important gaps: optimization of dissection techniques and development of a model of degeneration. Lee et al. [27], removed vertebral endplates to retain NP cell viability for 1 week, while other studies retained vertebral endplates through heparinization and cleaning to maintain live cells for 3 weeks [8, 22]. Yet optimal dissection procedures of large animal organ culture are still being developed and there are also very few IVD explant models of degeneration. The present work describes a bovine disc organ culture system, which permits the maintenance of cell viability for at least 4 weeks using a novel dissection technique, and in which degenerative changes can be induced as a prelude to studying repair.

Materials and methods

Source of reagents

The polyclonal rabbit antibody recognizing human chondroadherin was a kind gift from Dr. Dick Heinegård, Lund University, Sweden [25]. The polyclonal rabbit antibody recognizing the G1 region of human aggrecan was raised against synthetic peptides, as described previously [46]. The horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was from Santa Cruz Biotechnology. The enhanced chemiluminescence (ECL) detection system was from GE Healthcare. Keratanase II and chondroitinase ABC were purchased from BioLynx Inc.

Disc isolation

The largest first 4 caudal discs were isolated from the tails of 24- to 30-month-old steers within 18 h after slaughter. The tails were dissected free of skin, muscles and ligaments, and pedicles for each segment were removed. Whole discs were isolated using one of three techniques: with bony endplates (BEP), without endplates (NEP) or with cartilage endplates (CEP) (Fig. 1). For BEP and CEP, parallel axial cuts were made through the vertebral bodies close to the cartilage endplates. The CEP discs were then further processed so that the bone and the adjacent calcified part of the cartilaginous endplate were removed using a surgical round end boring bit on a high-speed drill (Racine, WI, USA). The discs were processed until the surface of

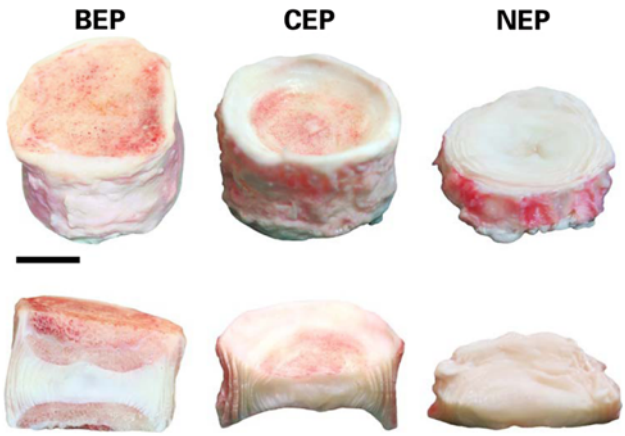

Fig. 1 Appearance of the IVDs isolated by three different methods. Images of the intact discs (*top row*) and of disc cut sagittally (*bottom row*) are shown. *BEP* disc retaining cartilage endplates plus adjacent vertebral bone, *CEP* disc retaining only cartilage endplates, and *NEP* disc without endplates. The *scale bar* represents 1 cm

the tissue was soft and flexible without detectable calcified tissue. NEP discs were isolated by parallel cuts using a straight edged microtome blade to fully remove both bone and cartilage endplates. The processed discs ranged from 27.6 ± 2.8, 29.5 ± 2.3 and 27.6 ± 1.5 mm in diameter and 4.4 ± 1.2, 16.2 ± 3.1, and 7.0 ± 1.0 g in weight for NEP, BEP and CEP discs, respectively

Disc organ culture

BEP discs were washed extensively with phosphate-buffered saline (PBS) containing 50 mM citrate to eliminate any blood clots and then followed the general procedure. BEP, NEP and CEP discs were rinsed in PBS supplemented with 1,000 U/mL penicillin, 1,000 μg/mL streptomycin (Gibco) and 0.25 μg/mL fungizone (Gibco), then placed in culture chambers (sterile 80 mL specimen containers, STARPLEX Scientific) containing 50 mL culture medium (Dulbecco's Modified Eagle Medium with 2 mM Glutamax and 25 mM Hepes, supplemented with 5% fetal bovine serum, 500 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL L-ascorbate). To evaluate cell viability the discs were cultured without external load applied for up to 4 weeks ($n = 8$ per time point). Medium was aspirated and replenished using a pipet-aid with a 25 mL pipet every 3 days over the entire culture period. To evaluate tissue swelling, discs isolated using the NEP or CEP method were placed in culture medium and weights were recorded after 15 min, 30 min, 1, 2, 4, 20, 25 and 30 h ($n = 4$ per group were tested)

Induced disc degeneration

Degeneration was induced by a single injection of trypsin (Sigma, 2.5 μg/50 μL PBS) into the center of the CEP disc using a 28-G needle after a 48 h pre-culturing period. Control discs were injected with the same volume of PBS.

Cell viability

A 1 mm section was taken through the center of the discs using an in-house designed cutting tool consisting of two microtome blades placed 1 mm apart. This gives a 1-mm-thick slice though the entire disc that is about 3 cm wide (disc diameter) and 1 cm high (disc height). The 1 mm tissue section was incubated in serum-free medium containing fluorescent dyes (Live/Dead®, Invitrogen) according to the manufacturer's instructions, using 1 mM each of calcein (494/517 nm) and ethidium homodimer-1 (528/617 nm, in the presence of DNA). The cells were visualized using an inverted confocal laser scanning microscope (CLSM, Zeiss LSM 510). 20 consecutive 6 μm sections were imaged. CLSM stacks were split into single images. The first image to be analyzed was chosen approximately five images into the stack to prevent artefacts from the cut surface and then extending another four images. The selected five images were merged, saved as single color JPEG files (red and green separate), and the labeled cells were quantified separately using the CellC software (Matlab) [44]. The live to dead cell ratio was then calculated from the proportion of green and red cells.

Extraction of ECM proteins and proteoglycans

NP and AF tissue ($n = 8$ per group) was excised separately and sliced thinly. Proteins and proteoglycans were extracted at 4°C under continuous agitation for 48 h on a wet weight per volume basis using 15 volumes extraction buffer [4 M guanidinium chloride, 50 mM sodium acetate, pH 5.8, 10 mM EDTA, COMPLETE® (Roche)]. The extracts were then cleared by centrifugation at 16,000*g* for 30 min.

GAG analysis

Sulfated glycosaminoglycans (GAGs) were quantified ($n = 8$ per group) from tissue extracts by a modified dimethyl methylene blue (DMMB) dye-binding assay [1]. Samples were diluted to fall in the middle of the linear range of the standard curve.

Western blot analysis

Proteins and proteoglycans in 5 μL aliquots of disc extracts were precipitated by the addition of 9 volumes of ethanol, washed twice in 95% ethanol, and finally lyophilized. Samples for analysis of CHAD were re-dissolved in SDS sample buffer. Samples for analysis of aggrecan were digested with keratanase II (Seikagaku) at 0.1 mU per 5 μL extract for 6 h. The solution was then adjusted to 100 mM Tris, 100 mM sodium acetate, pH 7.3, and digested overnight with chondroitinase ABC (Seikagaku) at 1 mU per 5 μL extract and then mixed with SDS sample buffer. Samples were separated by SDS-PAGE (10% or 4–12% NOVEX® gels) under reducing conditions. Separated proteins were transferred to nitrocellulose membranes [47], and western blotting was performed using antibodies recognizing chondroadherin and aggrecan. The membranes were blocked with 3% skim milk powder in 10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, and 0.2% Tween (blocking buffer), and then incubated with the primary antibodies at a 1:1,000 dilution in blocking buffer containing 3% BSA, followed by the secondary antibody conjugated with HRP (1:1,000 dilution) in blocking buffer containing 1% dried skim milk. The bound antibody was visualized by chemiluminescence (GE Healthcare).

Proteoglycan synthesis

CEP discs (7 days after trypsin-induced degeneration) were injected with 50 µL PBS containing 20 µg soya bean trypsin inhibitor (Sigma), 25 µCi ^{35}S (Perkin-Elmer), with or without 30 ng TGFβ1 (PreproTech Inc). NP tissue was collected 72 h after treatment and digested with proteinase K [9]. Aliquots of the proteinase K digests were dialyzed against 1 mM sodium sulfate in distilled water (BRL micro dialysis chamber, with SpectraPor 12–14 kDa molecular weight cut off dialysis membrane) to remove unincorporated ^{35}S-sulfate. Incorporated radioactivity was measured by scintillation counting and normalized to tissue wet weight ($n = 7$ per group).

Histology

A 1 mm section was taken through the center of the disc as described above. The intact slice was fixed between porous platens in 4% paraformaldehyde, lysine, periodate as described by McLean and Nakane [33] for 24 h before transferring to decalcifying solution (10% EDTA in 0.1 M Tris–HCL, pH 7.4) for 12 days with solution changes twice daily. Samples were then embedded in paraffin wax and 5-µm-thick sections were cut and stained with hematoxylin and Safranin O-fast green [42].

Statistical analysis

An unpaired t test was used to calculate significance in Figs. 2, 3, 4 and 9. In Figs. 5 and 7, multivariate linear models were used to assess the effects of treatment (trypsin vs. buffer injection) and time (4, 8 and 14 days, respectively). The value at time zero of native discs for DMMB and % live cells was used as an internal comparison to normalize all subsequent values. A p value of <0.05 was used as the criteria for a significant difference. Where multiple tests were performed, Bonferroni corrections were applied.

Results

Three isolation methods were compared to find a protocol allowing for long-term culture of IVDs which maintained cell viability, morphology and integrity of the tissue. The discs were cultured without external load, as optimal loading conditions will vary for discs prepared by each isolation technique and this would further complicate the comparison. As the NEP isolation technique severs the attachment of collagen fibrils between the disc and the cartilage endplates, it was predicted that NEP discs would be prone to swell to a greater extent than the CEP or BEP discs when maintained in culture. The swelling capacity of discs prepared with the CEP method was therefore compared to discs isolated using the NEP method. Increase in disc weight as an indicator of swelling was measured over a 30-h period (Fig. 2a). In both cases weight increase occurred rapidly during the first 4 h of culture, and then gradually over the next 26 h. The difference in swelling was already significant after 15 min ($p < 0.002$) and remained significant at all subsequent time points. Weight increase was limited to about 20% using the CEP method. In contrast, discs isolated using the NEP method increased between 60 and 100% of their initial weight when cultured unconfined. The CEP discs maintained their morphology throughout the experiment, whereas the NEP discs were already deformed after 1 h and were severely deformed after 30 h (Fig. 2b).

As excessive swelling of the disc, as occurs following the NEP isolation procedure, or impaired nutrient flow, as expected following the BEP procedure, may be detrimental to disc cell viability; cell survival in culture following the different disc isolation techniques was investigated. Discs prepared by the NEP and BEP methods were cultured for up to 10 days and cell viability was measured using the

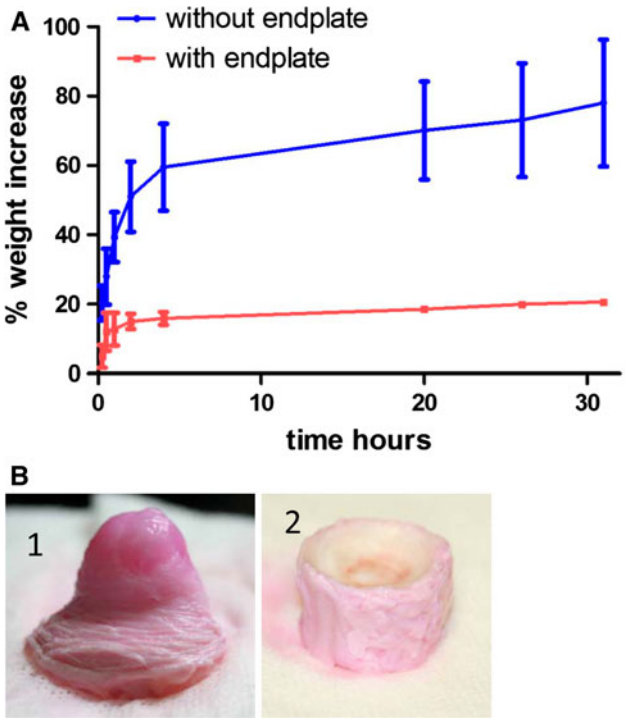

Fig. 2 Comparison of swelling capacity and deformation of CEP and NEP isolated discs. **a** CEP and NEP isolated discs were placed in culture medium and their weights were measured and plotted as % increase in weight over time ($n = 4$, *error bars* indicate standard deviation). CEP and NEP discs were compared at each time point using an unpaired t test, and p values <0.01 were obtained at all time points relative to time 0. **b** Deformation was compared between discs isolated by the NEP (*1*) and CEP (*2*) methods at 30 h

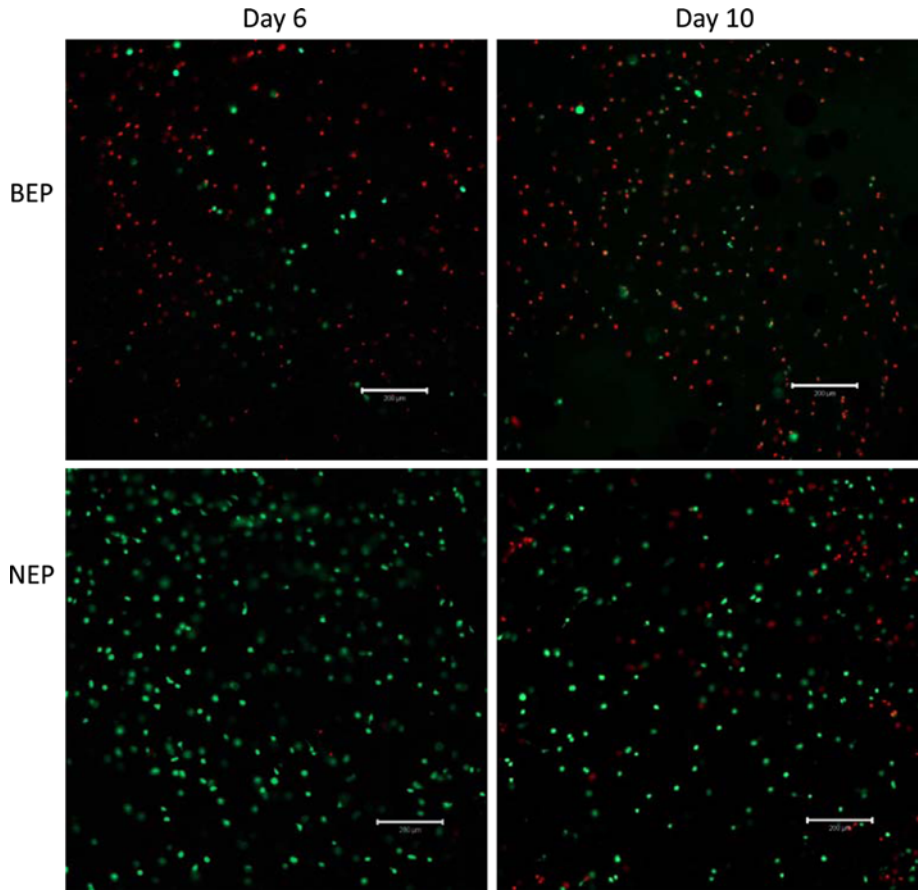

Fig. 3 Cell survival in the NP of BEP and NEP isolated discs. Cell viability was measured in a 1 mm thick tissue section after incubation in serum-free medium containing fluorescent dyes (Live/Dead®, Invitrogen). Live (*green*) and dead (*red*) cells were visualized using an inverted confocal laser scanning microscope after 6 or 10 days of culture ($n = 4$). The *scale bars* represent 200 μm

live/dead fluorescent system in combination with confocal microscopy. Although the NEP discs were severely deformed, cell survival was maintained with more than 75% viable cells in the NP throughout the culture period (day 6, 88 ± 28% and day 10, 84 ± 19%, this was not significant and gave p values of 0.7 and 0.6, respectively compared to freshly isolated discs). In contrast, while the morphology was retained in BEP discs, cell survival was compromised after 6 days of culture (43 ± 28%, $p = 0.04$ compared to freshly isolated discs) and the majority of the cells were dead after 10 days (27 ± 23% viable cells in all areas, $p = 0.004$ compared to freshly isolated discs) (Fig. 3). Because of these limitations, experiments with NEP and BEP isolated discs were not perused past day 10. Using the CEP method, which also allows maintenance of disc morphology but lacks the impediment to nutrition caused by the bony endplates, cell survival of the disc was maintained for up to 4 weeks (80 ± 7% in freshly isolated, 80 ± 13% at 2 weeks and 75 ± 12% at 4 weeks in all areas, this gave p values of 1 and 0.5 when compared to freshly isolated discs) (Fig. 4).

As the CEP method preserved both cell viability and morphology, it was chosen to develop a proteinase-induced model of disc degeneration. Trypsin was chosen for this purpose as it will degrade most ECM proteins but not the collagen fibrils. It also has very precise cleavage specificity and is readily inactivated by inhibitor treatment. In preliminary experiments, 0–5 μg of trypsin was injected into the center of CEP discs to determine the dose needed to deplete aggrecan from the NP to about 60–80% of its initial level. 2.5 μg trypsin per 50 μL buffer was selected for further study. When a single 2.5 μg trypsin injection was delivered to the center of the NP, GAG levels in the NP were depleted by about 40% after 4 days and by about 70% after 14 days (Fig. 5). Slow GAG loss also occurred in the control discs with a 25% depletion after 14 days. DMMB dropped significantly over time for both treatment groups ($p = 0.0006$). Trypsin-injected discs however were losing proteoglycans much faster. This was highly significant ($p < 0.0001$). Post hoc t tests comparing individual group means for proteoglycan concentrations with the native control at time zero were significantly lower (all $p < 0.01$), except for the buffer-injected discs at 4 days ($p = 0.74$). As expected GAG content in AF tissue was initially lower than in the NP. However, GAG loss in the AF of control and trypsin-treated discs occurred at a similar rate to that observed in the NP, indicating diffusion of the trypsin throughout the disc (data not shown). Safranin O staining of histological sections showed similar proteoglycan levels in freshly isolated and control discs cultured for 14 days

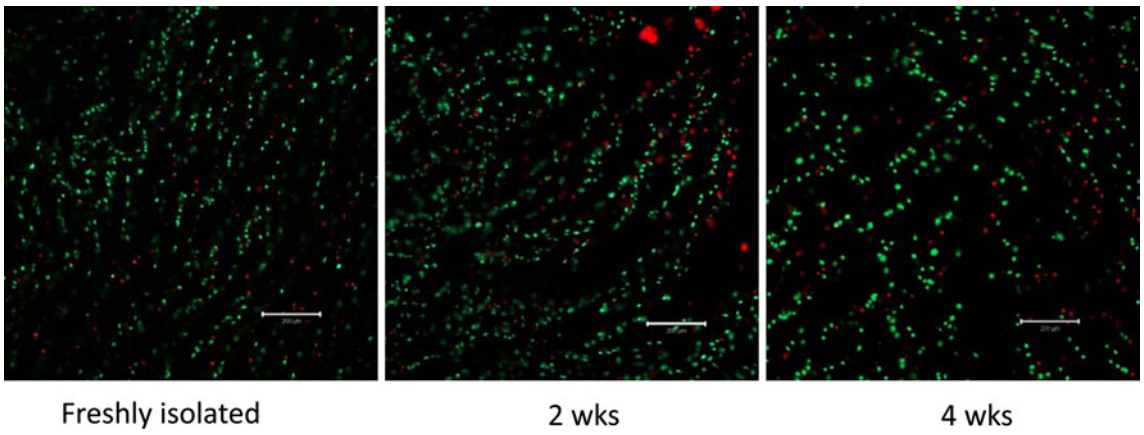

Fig. 4 Cell survival in the NP of CEP isolated discs. Cell viability was determined in a 1-mm-thick tissue section after incubation in serum-free medium containing fluorescent dyes (Live/Dead®, Invitrogen). Live (*green*) and dead (*red*) cells were visualized using an inverted confocal laser scanning microscope in freshly isolated discs and in discs cultured for 2 and 4 weeks ($n = 4$). The *scale bars* represent 200 μm

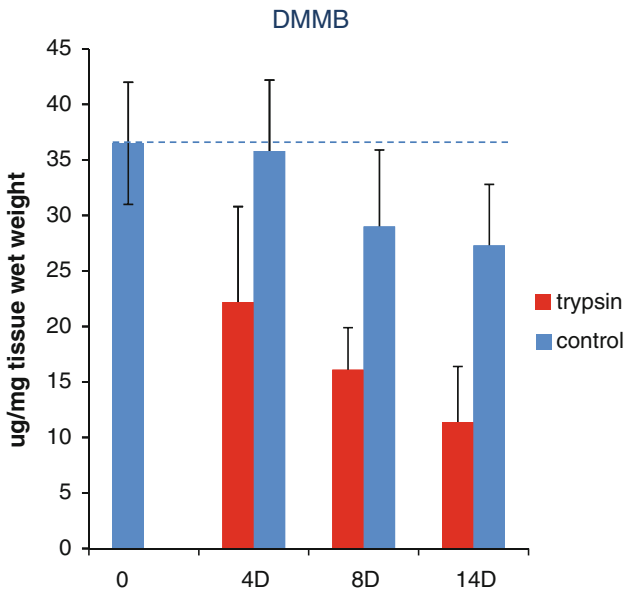

Fig. 5 Glycosaminoglycan content in CEP discs with or without trypsin treatment. Sulfated GAGs were quantified after trypsin or buffer alone was injected into the center of the disc. GAG content was measured in NP tissue of freshly isolated discs (0) and in discs 4, 8 and 14 days after injection ($n = 8$, *error bars* indicate standard deviation). Multivariate linear models were used to assess the effects of treatment, p values <0.001 in trypsin-treated sample and >0.05 in control samples, compared to time 0

and a loss of Safranin O staining in trypsin-treated discs at day 14 (Fig. 6), thus confirming the results obtained by the GAG assay. This method is only able to detect a significant difference in proteoglycan concentration and is not able to detect low levels of proteoglycans [42].

As trypsin treatment and the consequent extracellular matrix depletion has the potential to adversely affect cell viability, the proportion of live to dead cells was compared in trypsin-treated and control discs at different times in culture (Fig. 7a). Of the cells present in the tissue, an average cell survival rate greater than 75% was measured at all time points independent of manipulation (Fig. 7b), and no decline was observed with time in culture. The multivariate linear model failed to demonstrate any effect (adjusted $r^2 = 2\%$) for either treatment or time ($p = 0.40$ and 0.23, respectively). There was also no interaction found between treatment and time (treatment × time cross product p value = 0.33). Individual t tests comparing the group means for % live cells with the native control at time zero and applying Bonferroni corrections for multiple tests, all showed no significant differences. Individual t tests comparing the group means for % live cells with the native control at time zero, and applying Bonferroni corrections for multiple tests (i.e., the p level of 0.05 was corrected for 6 concurrent t tests, resulting in a more strict p level of $0.0500/6 = 0.0083$) all showed no significant differences. Thus, trypsin treatment is not detrimental to cell survival.

To determine the influence of trypsin on the extent of ECM proteoglycan and protein degradation, aggrecan and CHAD were analyzed by immunoblotting in extracts of the cultured discs. The profile of aggrecan showed little change after 8 days of culture in control discs. Of particular note is the absence of G1 region (MW 60–80 kDa) accumulation, which would be indicative of proteolysis. Thus, proteoglycan loss in the control cultures is probably due to passive diffusion rather than an active catabolic process. In contrast, there was extensive degradation of aggrecan following trypsin treatment, with the accumulation of fragments representing free G1 regions (Fig. 8a). In comparison to aggrecan, CHAD was not affected to the same extent by the trypsin treatment (Fig. 8b). There was a 25% depletion in CHAD, but there was no evidence for the

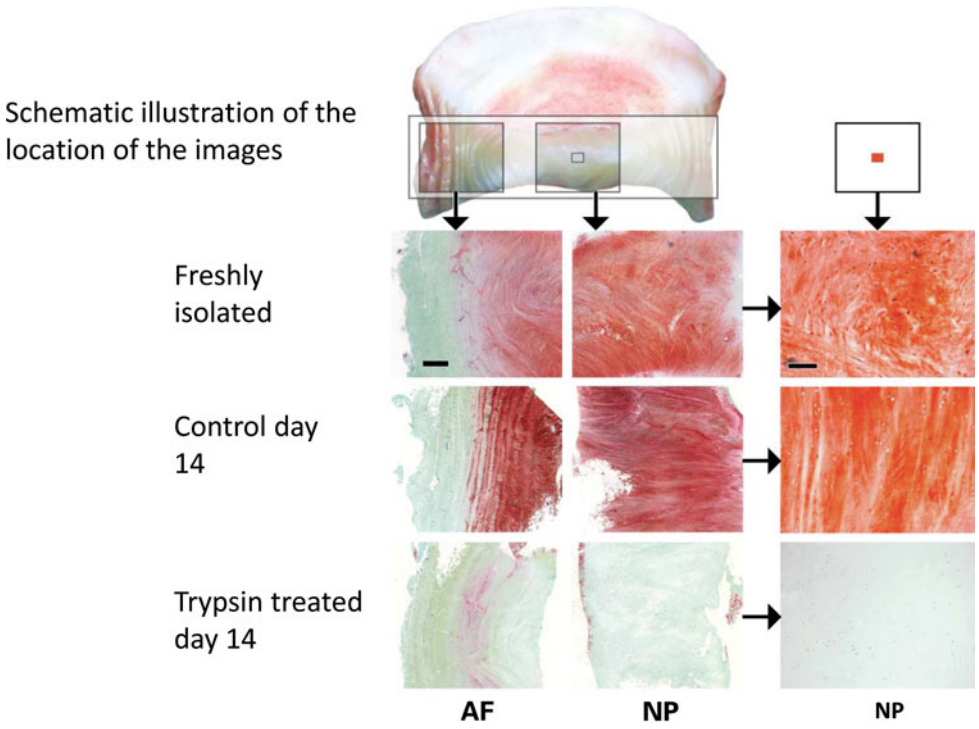

Fig. 6 Hematoxylin–Safranin O-fast green stained disc sections. Sagittal sections of AF and NP from freshly isolated discs and trypsin or buffer-injected CEP discs at day 14 are shown. The *scale bars* represent 2 mm for the left hand sections and 200 μm for other sections

accumulation of degradation products. These results suggest that while aggrecan has undergone extensive degradation and loss following trypsin treatment, the collagen network and its associated CHAD remain relatively intact. Such ECM changes are a feature of early disc degeneration.

TGFβ is known to stimulate proteoglycan synthesis in connective tissue cells [12], and was used to validate that the trypsin-treated discs can be used to evaluate a repair response following injection of a bio-active substance. TGFβ or buffer alone was injected into the center of the disc, together with radio-labeled sulfate and soya bean trypsin inhibitor, 1 week after trypsin-induced degeneration. The amount of newly synthesized proteoglycans 72 h after injection nearly doubled in TGFβ-treated discs ($p = 0.02$) (Fig. 9) in comparison to discs treated with buffer alone.

Discussion

Maintenance of the IVD matrix is orchestrated by its sparse cell population that controls the balance between anabolic and catabolic processes. This balance may be perturbed towards catabolic degeneration with age, as cell number and nutrition decline, and cell senescence increases [23, 26, 39]. Degeneration may also be enhanced by genetic differences, high magnitude or frequency loading of the disc, as well as by trauma to the disc [18, 28]. Independent of cause, once degenerative changes have been established, the adult IVD seems incapable of intrinsic repair. It is also not clear if repair can be induced by supplementation with anabolic agents in degenerate human discs. However, promising results have been demonstrated in discs from small animals using growth factor supplementation [7, 30]. Unlike humans, the discs of smaller animals maintain their notochordal cells throughout life, and these cells respond well to growth factors by the production of proteoglycans and the down-regulation of cytokines [31]. While promising results demonstrating an increase in proteoglycan production and mRNA expression have been demonstrated in isolated human disc cells [20], it is important to verify the findings in a system where the cells are in their native three-dimensional environment. As in vivo work in the human is not feasible, and large animals with a disc structure similar to humans are expensive and difficult to maintain, the availability of an intact organ culture system applicable to human discs would seem ideal.

The objective of this study was to develop a whole disc organ culture system using bovine IVDs. Such discs are of similar structure to humans and possess the same barriers to nutrient diffusion, but have the advantage of being readily available and not having variable degrees of degeneration. The bovine discs were isolated using three different techniques, and were cultured without external load. We chose to develop a system where no external load is being applied, as the frequency and magnitude of optimal loading might differ for the different isolation

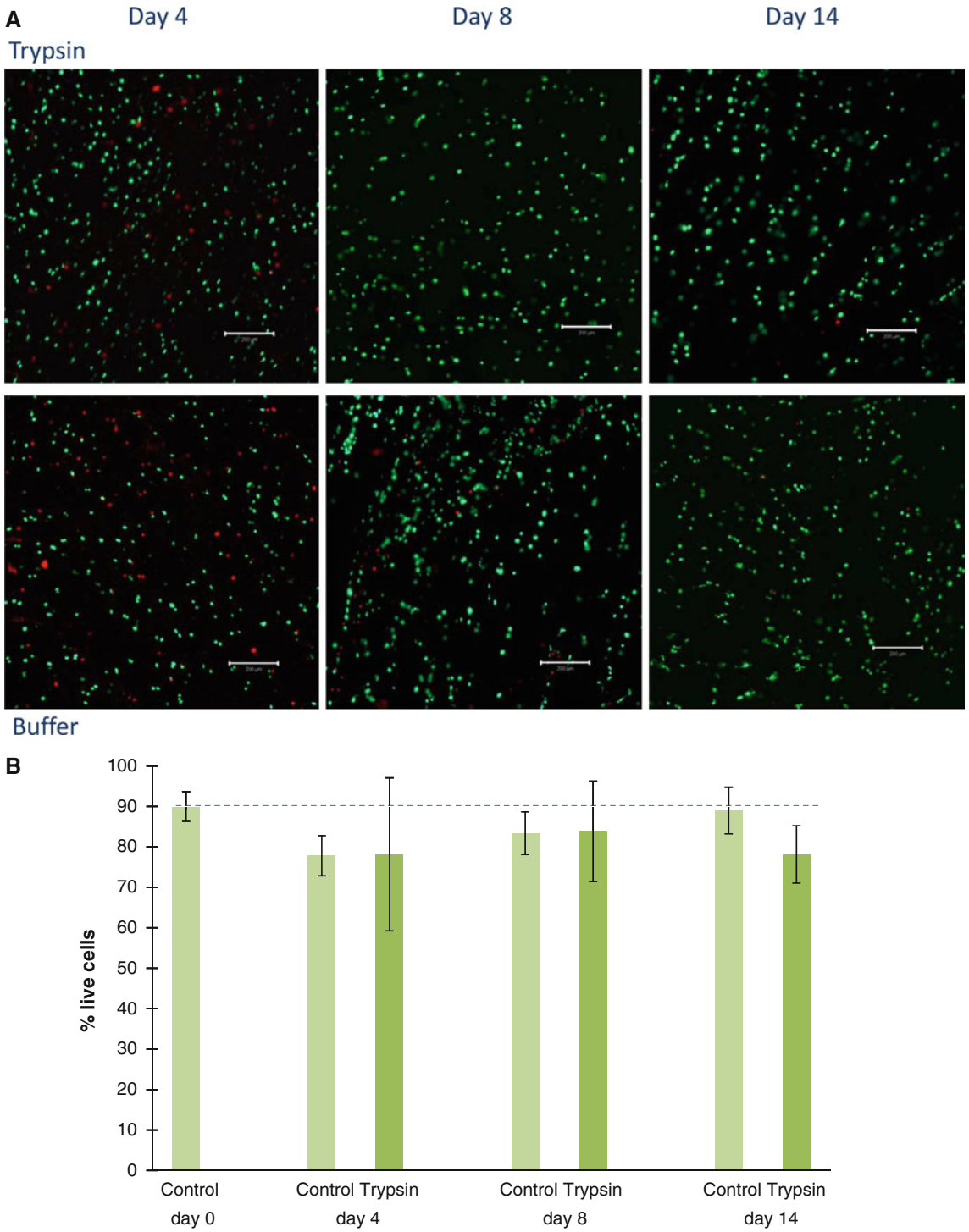

Fig. 7 Cell viability in trypsin and buffer injected CEP discs. **a** Cell viability was evaluated using fluorescent dyes (Live/Dead®, Invitrogen), and live (*green*) and dead (*red*) cells were visualized using an inverted confocal laser scanning microscope in trypsin and control-injected discs at 4, 8 and 14 days after treatment. The *scale bars* represent 200 μm. **b** The proportion of live to dead cells was quantified separately in 5 selected, 6 μm sections using the CellC software. Cell viability was calculated as a live to dead cell ratio and presented as % live cells ($n = 8$, *error bars* indicate standard deviation). Multivariate linear models were used to assess the effects of treatment, no significant difference was found compared to the control day 0, $p > 0.05$)

techniques further complicating the comparison. Cell survival was not drastically changed in discs isolated without any cartilaginous or bony endplates over a 2 week period, but their enormous swelling and severe deformation made them unsuitable for studying repair. On the other hand, discs cultured with intact bony endplates preserved their

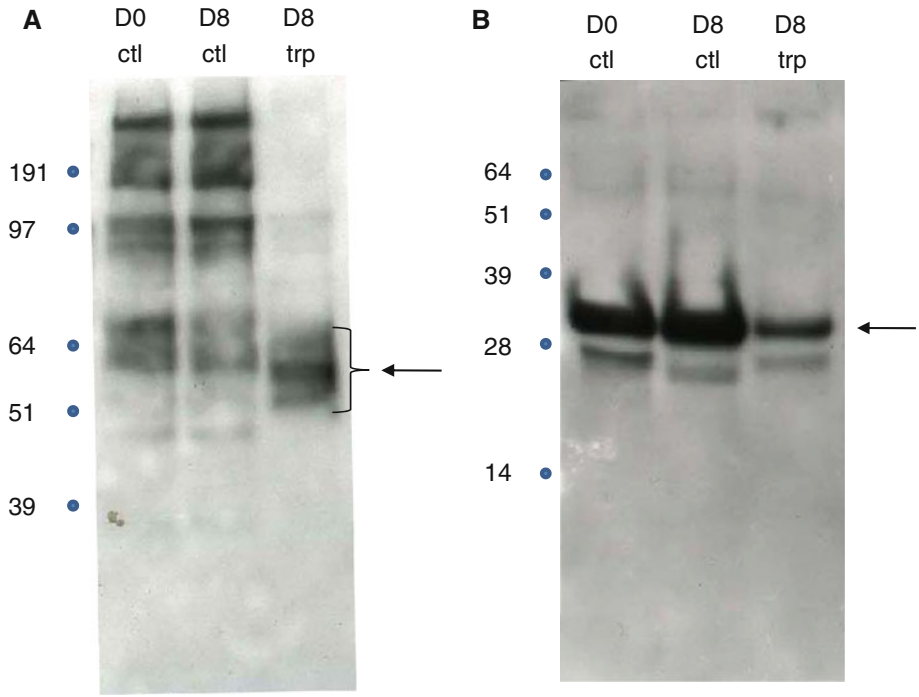

Fig. 8 ECM and proteoglycan degradation caused by a single trypsin injection. Proteoglycan and protein degradation were analyzed by SDS-PAGE and western blotting using antibodies recognizing aggrecan (**a**) and CHAD (**b**). Pooled extract from 8 discs was analyzed in freshly isolated NP tissue (*ctl*), as well as in NP tissue 8 days after trypsin (*D8 trp*) or buffer injection (*D8 ctl*). Fragments representing free aggrecan G1 region or intact CHAD are indicated with an *arrow*

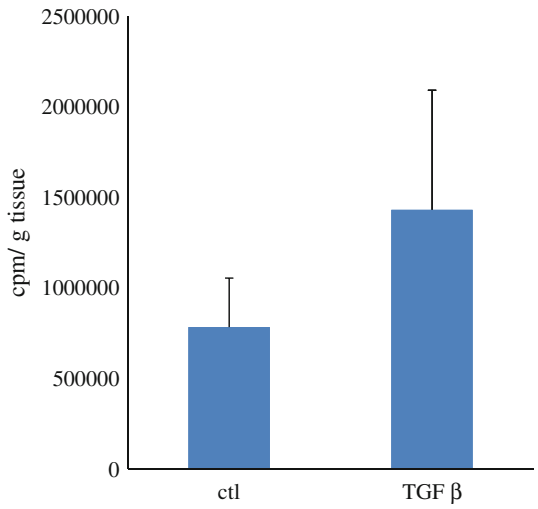

Fig. 9 Proteoglycan synthesis in trypsin and buffer-injected CEP discs following TGFβ injection. Incorporation of radiolabeled sulfate was quantified 72 h after injection of 30 ng TGFβ (*TGF* β) or buffer alone (*ctl*). Sulfate incorporation is indicated as cpm per gram wet weight tissue ($n = 7$ TGFβ injected and $n = 8$ control discs per group, *error bars* indicate standard deviation). An unpaired *t* test gave a *p* value of 0.02

integrity and shape, but cell survival was compromised and most of the cells were dead after 10 days in culture, also making it impossible to study repair in this system. In contrast, discs isolated with intact cartilaginous endplates overcame these shortcomings, as both disc integrity and cell viability were maintained. It is likely that with the bony endplate present nutrient supply to the cells in the center of the disc is severely impaired, but that retention of only the non-calcified cartilaginous endplate allows adequate nutrition to be maintained. While the discs maintained their gross morphology with the cartilaginous endplates present, their weight did increase by up to 20%. However, the expected increase in disc height with such limited swelling is within the reported diurnal variation range [38]. This may be a major contributor to the retention of cell viability, and may ensure that normal cell metabolism is maintained.

Organ cultures of whole bovine discs have been reported previously using the BEP and NEP methods [27]. The discs were maintained under a constant load to negate the effects of swelling, and cultures were maintained for 7 days. At the end of this time the majority of cells in the BEP discs were dead, whereas good cell viability was obtained for the NEP discs. The difference between the two isolation techniques presumably reflects the decreased nutrition to the discs when the bony endplate remains intact. The work also illustrates that the NEP method of isolation may be of value if the resulting discs are maintained under load; however, additional studies have shown that the type of loading is important if cell death is to be avoided [24]. The BEP isolation method has been shown to be of use for viable organ culture when the discs were obtained from sheep that were heparinized prior to slaughter [8, 22]. Presumably, heparinization prevented blood clot formation in the capillary channels that are present in the endplate, and so improved nutrient flow. This technique would not however be a feasible option for bovine discs obtained from the abattoir, and this is by far the most convenient source for large animal discs in large quantity. The use of

the CEP isolation technique avoids the need for heparinization to improve nutrient supply to the disc, and also avoids the need for the use of loading devices to prevent excessive swelling that is inherent to the NEP method, as the CEP discs are being contained by the intact cartilage endplates which limit swelling. However, the CEP discs are also amenable to cyclic and higher static loading should this be desired.

Roberts et al. [40] have previously described a technique for developing an explant model of disc degeneration in the bovine caudal disc that would be suitable for testing injectable nucleus pulposus replacements. In this work 100 times more trypsin compared to our study was used and cell viability was not assessed. This resulted in a depletion of the entire matrix and cells in the center of the discs, and created a cavity where cell-containing scaffolds can be injected. This model would probably compare to late stages of disc degeneration and the method would be of limited use to study the effect of bioactive substances on cells already present in the disc The model that we have developed represents an earlier stage of disc degeneration where existing cells can be stimulated to replace the proteoglycan content that is initially lost.

Currently, treatment of symptomatic disc degeneration often involves surgical intervention and long-term rehabilitation, and is only an option at late stages of degeneration [6, 10, 32]. As surgical intervention involves removal of the diseased intervertebral disc and fusing the adjacent vertebral bone, it leads to a loss of mobility of that joint, which can often result in degeneration at adjacent disc levels. New biological therapies targeted to intervertebral discs to prevent or retard degenerative changes and to promote repair could potentially alleviate or at least delay the need for surgical intervention. Biological treatment is most likely to be a feasible therapeutic option in early stages of disc degeneration, characterized by loss of proteoglycans but the maintenance of a relatively intact collagen network. In the present study, this type of early degenerative change was induced in normal bovine discs by injecting trypsin into their centers. As this induced degeneration occurs with the long-term retention of cell viability, and preserves metabolically active cells, it represents an ideal system in which to study subsequent repair induced by growth factors. In the present study TGFβ was injected into the trypsin-treated discs and proteoglycan synthesis was increased, demonstrating the feasibility of studying repair in this system. TGFβ was used to establish a proof of principle as it is known to induce proteoglycan synthesis in disc cells [43], but it may not be the most appropriate agent for therapeutic use in humans. An unloaded system was used for the present study and this is a valid first screening step as it allows for many replicates to be run simultaneously. Although, the application of physiological load would likely enhance matrix synthesis and thereby the repair response, any substance able to increase synthesis in an unloaded system could be considered as a suitable candidate to further test in a more complex loaded system and finally in a living animal.

Acknowledgments This work was supported by financial support from AOSpine grant number AOSBR-07-07, the Canadian Arthritis Network and the Shriners of North America.

Conflict of interest None.

References

1. Barbosa I, Garcia S, Barbier-Chassefiere V, Caruelle JP, Martelly I, Papy-Garcia D (2003) Improved and simple micro assay for sulfated glycosaminoglycans quantification in biological extracts and its use in skin and muscle tissue studies. Glycobiology 13:647–653
2. Bertram H, Steck E, Zimmerman G, Chen B, Carstens C, Nerlich A, Richter W (2006) Accelerated intervertebral disc degeneration in scoliosis versus physiological ageing develops against a background of enhanced anabolic gene expression. Biochem Biophys Res Commun 342:963–972
3. Buckwalter JA (1995) Aging and degeneration of the human intervertebral disc. Spine 20:1307–1314
4. Camper L, Heinegård D, Lundgren-Åkerlund E (1997) Integrin alpha2beta1 is a receptor for the cartilage matrix protein chondroadherin. J Cell Biol 138:1159–1167
5. DiFabio JL, Pearce RH, Caterson B, Hughes H (1987) The heterogeneity of the non-aggregating proteoglycans of the human intervertebral disc. Biochem J 244:27–33
6. Diwan AD, Parvataneni HK, Khan SN, Sandhu HS, Girardi FP, Cammisa FP Jr (2000) Current concepts in intervertebral disc restoration. Orthop Clin North Am 31:453–464
7. Ellman MB, An HS, Muddasani P, Im HJ (2008) Biological impact of the fibroblast growth factor family on articular cartilage and intervertebral disc homeostasis. Gene 420:82–89
8. Gantenbein B, Grunhagen T, Lee CR, van Donkelaar CC, Alini M, Ito K (2006) An in vitro organ culturing system for intervertebral disc explants with vertebral endplates: a feasibility study with ovine caudal discs. Spine 31:2665–2673
9. Gooch KJ, Blunk T, Courter DL, Sieminski AL, Vunjak-Novakovic G, Freed LE (2002) Bone morphogenetic proteins-2, -12, and -13 modulate in vitro development of engineered cartilage. Tissue Eng 8:591–601
10. Guyer RD, Foley KT, Phillips FM, Ball PA (2003) Minimally invasive fusion: summary statement. Spine 28:S44
11. Haglund L, Ouellet J, Roughley P (2009) Variation in chondroadherin abundance and fragmentation in the human scoliotic disc. Spine 34:1513–1518
12. Hardingham TE, Bayliss MT, Rayan V, Noble DP (1992) Effects of growth factors and cytokines on proteoglycan turnover in articular cartilage. Br J Rheumatol 31(Suppl 1):1–6
13. Heathfield SK, Le Maitre CL, Hoyland JA (2008) Caveolin-1 expression and stress-induced premature senescence in human intervertebral disc degeneration. Arthritis Res Ther 10:R87
14. Horner HA, Urban JP (2001) Effect of nutrient supply on the viability of cells from the nucleus pulposus of the intervertebral disc. Spine 26:2543–2549

15. Humzah MD, Soames RW (1988) Human intervertebral disc: structure and function. Anat Rec 220:337–356
16. Hunter CJ, Matyas JR, Duncan NA (2003) The notochordal cell in the nucleus pulposus: a review in the context of tissue engineering. Tissue Eng 9:667–677
17. Hunter CJ, Matyas JR, Duncan NA (2004) The functional significance of cell clusters in the notochordal nucleus pulposus: survival and signaling in the canine intervertebral disc. Spine 29:1099–1104
18. Hutton WC, Toribatake Y, Elmer WA, Ganey TM, Tomita K, Whitesides TE (1998) The effect of compressive force applied to the intervertebral disc in vivo. A study of proteoglycans and collagen. Spine 23:2524–2537
19. Iatridis JC, Mente PL, Stokes IA, Aronsson DD, Alini M (1999) Compression-induced changes in intervertebral disc properties in a rat tail model. Spine 24:996–1002
20. Imai Y, Miyamoto K, An HS, Thonar EJ, Andersson GB, Masuda K (2007) Recombinant human osteogenic protein-1 upregulates proteoglycan metabolism of human anulus fibrosus and nucleus pulposus cells. Spine 32:1303–1309
21. Jahnke MR, McDevitt CA (1988) Proteoglycans of the human intervertebral disc. Electrophoretic heterogeneity of the aggregating proteoglycans of the nucleus pulposus. Biochem J 251:347–356
22. Junger S, Gantenbein-Ritter B, Lezuo P, Alini M, Ferguson SJ, Ito K (2009) Effect of limited nutrition on in situ intervertebral disc cells under simulated-physiological loading. Spine 34:1264–1271
23. Kletsas D (2009) Senescent cells in the intervertebral disc: numbers and mechanisms. Spine J 9:677–678
24. Korecki CL, MacLean JJ, Iatridis JC (2007) Characterization of an in vitro intervertebral disc organ culture system. Eur Spine J 16:1029–1037
25. Larsson T, Sommarin Y, Paulsson M, Antonsson P, Hedbom E, Wendel M, Heinegård D (1991) Cartilage matrix proteins. A basic 36-kDa protein with a restricted distribution to cartilage and bone. J Biol Chem 266:20428–20433
26. Le Maitre CL, Freemont AJ, Hoyland JA (2007) Accelerated cellular senescence in degenerate intervertebral discs: a possible role in the pathogenesis of intervertebral disc degeneration. Arthritis Res Ther 9:R45
27. Lee CR, Iatridis JC, Poveda L, Alini M (2006) In vitro organ culture of the bovine intervertebral disc: effects of vertebral endplate and potential for mechanobiology studies. Spine 31:515–522
28. Lotz JC, Hsieh AH, Walsh AL, Palmer EI, Chin JR (2002) Mechanobiology of the intervertebral disc. Biochem Soc Trans 30:853–858
29. Månsson B, Wenglén C, Mörgelin M, Saxne T, Heinegård D (2001) Association of chondroadherin with collagen type II. J Biol Chem 276:32883–32888
30. Masuda K (2008) Biological repair of the degenerated intervertebral disc by the injection of growth factors. Eur Spine J 17(Suppl 4):441–451
31. Masuda K, An HS (2006) Prevention of disc degeneration with growth factors. Eur Spine J 15(Suppl 3):S422–S432
32. Mayer HM, Wiechert K, Korge A, Qose I (2002) Minimally invasive total disc replacement: surgical technique and preliminary clinical results. Eur Spine J 11 (Suppl 2):S124–S130
33. McLean IW, Nakane PK (1974) Periodate–lysine–paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J Histochem Cytochem 22:1077–1083
34. Melrose J, Smith S, Ghosh P (2000) Differential expression of proteoglycan epitopes by ovine intervertebral disc cells. J Anat 197(Pt 2):189–198
35. Mizuno M, Fujisawa R, Kuboki Y (1996) Bone chondroadherin promotes attachment of osteoblastic cells to solid-state substrates and shows affinity to collagen. Calcif Tissue Int 59:163–167
36. Neame PJ, Sommarin Y, Boynton RE, Heinegård D (1994) The structure of a 38-kDa leucine-rich protein (chondroadherin) isolated from bovine cartilage. J Biol Chem 269:21547–21554
37. Oegema TR Jr (2002) The role of disc cell heterogeneity in determining disc biochemistry: a speculation. Biochem Soc Trans 30:839–844
38. Roberts N, Hogg D, Whitehouse GH, Dangerfield P (1998) Quantitative analysis of diurnal variation in volume and water content of lumbar intervertebral discs. Clin Anat 11:1–8
39. Roberts S, Evans EH, Kletsas D, Jaffray DC, Eisenstein SM (2006) Senescence in human intervertebral discs. Eur Spine J 15(Suppl 3):S312–S316
40. Roberts S, Menage J, Sivan S, Urban JP (2008) Bovine explant model of degeneration of the intervertebral disc. BMC Musculoskelet Disord 9:24
41. Roberts S, Urban JP, Evans H, Eisenstein SM (1996) Transport properties of the human cartilage endplate in relation to its composition and calcification. Spine 21:415–420
42. Rosenberg L (1971) Chemical basis for the histological use of safranin O in the study of articular cartilage. J Bone Jt Surg Am 53:69–82
43. Roughley P, Hoemann C, DesRosiers E, Mwale F, Antoniou J, Alini M (2006) The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation. Biomaterials 27:388–396
44. Selinummi J, Seppala J, Yli-Harja O, Puhakka JA (2005) Software for quantification of labeled bacteria from digital microscope images by automated image analysis. Biotechniques 39:859–863
45. Shen Z, Gantcheva S, Månsson B, Heinegård D, Sommarin Y (1998) Chondroadherin expression changes in skeletal development. Biochem J 330:549–557
46. Sztrolovics R, Alini M, Roughley PJ, Mort JS (1997) Aggrecan degradation in human intervertebral disc and articular cartilage. Biochem J 326(Pt 1):235–241
47. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
48. Urban JP (2000) Role of mechanical constraints in the maintenance and degradation of articular cartilage. Rev Prat 50:9–12
49. Wang JC, Kabo JM, Tsou PM, Halevi L, Shamie AN (2005) The effect of uniform heating on the biomechanical properties of the intervertebral disc in a porcine model. Spine J 5:64–70