European Cells and Materials Vol. 28 2014 (pages 98-111) DOI: 10.22203/eCM.v028a08 ISSN 1473-2262

ACUTE MECHANICAL INJURY OF THE HUMAN INTERVERTEBRAL DISC: LINK TO DEGENERATION AND PAIN

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Abstract

Excessive mechanical loading or acute trauma to intervertebral discs (IVDs) is thought to contribute to degeneration and pain. However, the exact mechanisms by which mechanical injury initiates and promotes degeneration remain unclear. This study investigates biochemical changes and extracellular matrix disruption in whole-organ human IVD cultures following acute mechanical injury. Isolated healthy human IVDs were rapidly compressed by 5 % (non-injured) or 30 % (injured) of disc height. 30 % strain consistently cracked cartilage endplates, confirming disc trauma. Three days post-loading, conditioned media were assessed for proteoglycan content and released cytokines. Tissue extracts were assessed for proteoglycan content and for aggrecan integrity. Conditioned media were applied to PC12 cells to evaluate if factors inducing neurite growth were released. Compared to controls, IVD injury caused significant cell death. Injury also caused significantly reduced tissue proteoglycan content with a reciprocal increase of proteoglycan content in culture media. Increased aggrecan fragmentation was observed in injured tissue due to increased matrix metalloproteinase and aggrecanase activity. Injured-IVD conditioned media contained significantly elevated interleukin (IL)-5, IL-6, IL-7, IL-8, MCP-2, GROα, and MIG, and ELISA analysis showed significantly increased nerve growth factor levels compared to non-injured media. Injured-disc media caused significant neurite sprouting in PC12 cells compared to non-injured media. Acute mechanical injury of human IVDs *ex vivo* initiates release of factors and enzyme activity associated with degeneration and back pain. This work provides direct evidence linking acute trauma, inflammatory factors, neo-innervation and potential degeneration and discogenic pain *in vivo*.

Keywords: Intervertebral disc**,** mechanical injury, inflammation, extracellular matrix, degeneration, nerve growth factor.

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Introduction

Intervertebral disc (IVD) degeneration is a debilitating disease directly contributing to low back pain and accounts for tremendous healthcare costs world-wide (Manchikanti *et al.*, 2009). IVDs function to provide load-bearing and range of motion to the trunk, neck and head. IVDs are comprised of a gelatinous nucleus pulposus (NP) and the fibro-cartilaginous annulus fibrosus (AF), which forms concentric rings around the central NP (Roughley, 2004). The porous cartilaginous endplates (EPs) cap the disc both cranially and caudally acting as a barrier between disc tissue and the vertebral bodies, allowing nutrient and waste diffusion into and out of the disc. The extracellular matrix (ECM) of the disc is composed mainly of collagen and proteoglycan, which contribute to disc mechanical properties (Gardner-Morse and Stokes, 2004; Roughley, 2004). NP and AF cells are responsible for balancing anabolic and catabolic turnover towards ECM maintenance.

IVD degeneration occurs when there is a catabolic shift whereby NP and AF cells synthesise more matrix-degrading enzymes than ECM components. One hallmark of ECM degradation is cleavage and loss of the abundant proteoglycan aggrecan, which gives the NP its high water content and compressive resistance (Adams and Roughley, 2006). Ultimately, matrix degradation leads to a combination of IVD mechanical dysfunction, cell death and inflammation. Disc degeneration is thought to result from several factors including trauma, disc prolapse or herniation, genetic factors, lifestyle, smoking, and atherosclerosis (Kelsey *et al.*, 1984; Battie *et al.*, 1991; Videman *et al.*, 1995; Adams and Roughley, 2006). However, the exact events and initiating mechanisms of IVD degeneration are poorly understood.

The inflammatory response observed during IVD degeneration has been hypothesised to modulate degradation of the ECM during disease progression. Inflammatory factors released by disc cells can act to increase expression of matrix-degrading catabolic enzymes such as matrix metalloproteinases (MMP) and aggrecanases (ADAMTS) (Le Maitre *et al.*, 2005; Phillips *et al.*, 2013). Disruption of ECM integrity and loss in disc height or disc herniation can lead to nerve impingement and neuropathic pain (Freemont *et al.*, 2002). Studies have indicated that increased inflammatory factors can promote neurite outgrowth and sensitise neurons (Richardson *et al.*, 2012; Wuertz and Haglund, 2013; Gawri *et al.*, 2014; Krock *et al.*, 2014), which is directly related to painful disc degeneration in patients. Furthermore, annulus fissures

have been demonstrated to allow for a focal proteoglycan loss leading to nerve and blood vessel ingrowth which can cause pain (Stefanakis *et al.*, 2012). Pro-inflammatory and pro-nociceptive factors found to be increased in these patients include interleukin-1 beta (IL-1β), IL-6, IL-8, tumour necrosis factor alpha (TNF-α), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) (Freemont *et al.*, 2002; Purmessur *et al.*, 2008; Gruber *et al.*, 2012; Krock *et al.*, 2014). Interestingly, an inflammatory response can be activated by adverse mechanical stimulation at the cellular level (Gilbert *et al.*, 2010; Gawri *et al.*, 2014) as well as the intact disc level in animal models (Miyagi *et al.*, 2012). Vertebral endplate fracture has also been linked to increased catabolic enzyme and pro-inflammatory gene expression (Wang *et al.*, 2012; Fields *et al.*, 2014). Taken together, these recent studies strongly suggest a biomechanical role in initiation and progression of IVD inflammation, degeneration and pain.

Research investigating the role of inflammation in painful human disc degeneration has focused on analysis of surgically removed degenerate IVD samples or cadaveric IVD samples at various times post-mortem (Richardson *et al.*, 2009; Gruber *et al.*, 2010; Richardson *et al.*, 2012; Phillips *et al.*, 2013; Krock *et al.*, 2014), usually from tissue that is already degenerate. Several recent studies utilising *ex vivo* (Iatridis *et al.*, 2005; Walter *et al.*, 2011; Dudli *et al.*, 2012) and *in vivo* (Miyagi *et al.*, 2012) animal models have established a strong correlation between injurious mechanical loading in IVD degeneration. However, no studies to-date have been able to analyse in real-time the events underlying degeneration to otherwise healthy human whole-disc organ cultures – and it is therefore pertinent to generate a mechanical overload model of human IVDs to better link animal model data to human disease.

This study demonstrates the consequences of a single high-impact traumatic loading event on human intervertebral discs with intact cartilaginous endplates. Here, traumatic loading was applied to healthy human intervertebral discs, and cell viability, matrix disruption and inflammatory responses were assessed to determine if acute mechanical trauma *ex vivo* initiates events associated with IVD degeneration and pain.

Materials and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM), Tween20, BSA, Safranin-O, Fast Green, dimethylmethylene blue, rat tail collagen type I and Poly-L-Lysine were purchased from Sigma (Burlington, ON, Canada). LIVE/DEAD assay was purchased from Invitrogen (Eugene, OR, USA). Methanol was purchased from VWR (Radnor, PA, USA). EDTA-free protease inhibitor tablets were purchased from Roche (Indianapolis, IN, USA). Keratanase II and Chondroitinase ABC were purchased from AMS biotechnology (Lake Forest, CA, USA). Novex 4-12 % Tris-Glycine gradient gels were purchased from Life Technologies (Carlsbad, CA, USA). Carnation Skim Milk Powder was used to block membranes for Western blot (Markham, ON, Canada). Rabbit IgG secondary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). PC12 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, Antibiotic-Antimycotic Solution, Foetal Bovine Serum, and Horse Serum were purchased from Life Technologies (Gibco) (Carlsbad, CA, USA). Western Lightning Plus Electrochemiluminescence Kit was purchased from Perkin Elmer (Waltham, MA, USA). Recombinant Neuronal Growth Factor (bNGF) was purchased from BioShop (Burlington, ON, Canada). Human Cytokine Array 1 Map (product code: AAH-CYT-1), NGF ELISA (ELH-BNGF-001), BDNF ELISA (ELH-BDNF-001), and IL-1β ELISA (ELH-IL1BETA-001) were all purchased from RayBiotech (Norcross, GA, USA). Aggrecan antibodies recognising the G1 domain (G1), the matrix metalloproteinase (MMP)-generated neoepitope (MMP-neo) and the aggrecanase generated neoepitope (AGCN-neo) used for immunoblotting were prepared as described previously (Roughley *et al.*, 2012).

Tissue source

With approval of the McGill University institutional review board, and in collaboration with the provincially run organ donation program: Transplant Quebec, three human lumbar spines were harvested *en bloc* using a standardised sterile surgical technique, and a total of 2 discs per donor were obtained (age 21, 34 and 47 – Table 1), and each donor acted as its own control. Strict selection criteria were followed ensuring that only healthy IVD were selected for this study. All donors had no history of back-pain based on an in-house Transplant Quebec-approved questionnaire, and all spines were visually and radiographically examined for any signs of degeneration. The harvest procedure was performed within 6 h of clamping of the aorta. Discs were extracted from the lower lumbar segment (Table 1) and cultured leaving the cartilaginous endplates intact using a previously described culture method (Gawri *et al.*, 2011). Briefly, discs were isolated by parallel cuts in the adjacent vertebral bodies close (within 0.5 cm) to the cartilaginous

Table 1. Demographics of donors and disc levels used in the study.

Donor	Age (years)	Disc level	Compression	Surface area [cm^2]	Height at 100 N [cm]	Weight [g]
1	47	L 3-4	Non-injured	16.60	1.57	18.2
		L 4-5	Injured	17.01	1.64	20.1
2	21	T 11-12	Non-injured	10.30	1.05	10.4
		L 1-2	Injured	12.70	1.30	14,1
3	35	T 12 - L 1	Non-injured	14.10	1.74	30.3
		L 1-2	Injured	20.22	1.70	30.1

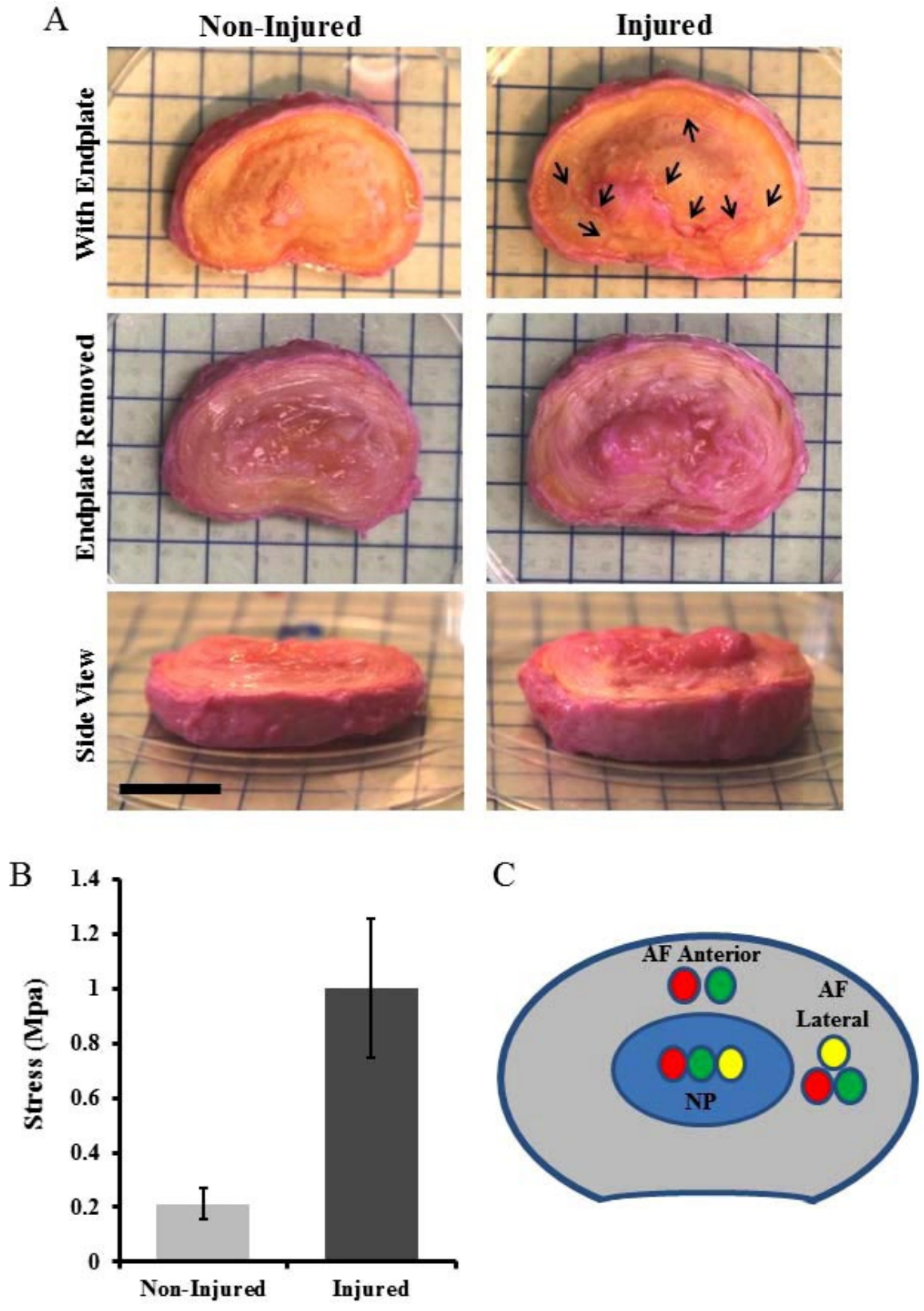

Fig. 1. A single-ramp compression consistently cracks cartilaginous endplates of healthy human IVDs. (**A**) Representative macroscopic view of IVD damage after non-injurious (5 % strain) and injurious (30 % strain) load. Top panels show discs with endplates. Lower panels show different views of the same samples with endplates removed (14 days post-loading). Arrows indicate large fissures in endplate. Scale bar represents 2 cm. (**B**) Measured average peak stress for non-injurious and injurious load. $n = 3$ for each group. Error bars indicate ± SD. (**C**) Schematic indicating where biopsy punches were taken from discs for further analysis. Red circles indicate samples taken for viability assays, green circles indicate samples taken for protein/proteoglycan extractions, and yellow circles indicate samples taken for histology.

endplate. A high speed bone burr (Series SR Foredom, Bethel, CT, USA) was used to remove all bone until only the cartilage endplate was remaining.

Following the termination of the 14 day culture period, the endplates were removed in order to macroscopically visualise damage to the underlying tissue following compression. Endplates were lifted at one lateral end with forceps and a surgical blade was used to dismember them from the disc tissue beneath. Initially, endplate was removed from the annulus fibrosus on all sides, and then proceeding to the remaining portion where the endplate covering the nucleus pulposus was removed, thereby allowing easier core punches to be taken for tissue analysis.

Loading protocol

Using a Mini Bionix 858 MTS machine, isolated discs were held under a mild load of 100 N for 2 min to assess disc height. Discs were then subjected to a single compression of either 5 % (non-injured) or 30 % (injured) strain at a rate of 30 % per second. Each donor had one disc serving as control and one serving as injured. Average peak stress achieved was 0.212 ± 0.056 MPa (non-injured) or 1.002 ± 0.256 MPa (injured) (Fig. 1B). 30 % injurious compression consistently yields endplate fracture (Fig. 1A, arrows). These particular loading schemes were chosen to simulate non-injurious (5 % – ~0.2 MPa) and acute hyperphysiological (30 % – ~1.0 MPa) compressions respectively. It also allowed the two groups to be manipulated in the same way with the only difference being load magnitude. Discs were immediately placed in 3.5 mL culture medium per gram of tissue (Gawri *et al.*, 2011) (DMEM containing 4.5 g/L glucose and supplemented with 1 % foetal calf serum (FCS), 25 mmol/L HEPES, 0.25 μg/mL fungizone, 50 μg/mL L-ascorbate, 2 mmol/L GlutaMAX and 50 μg/mL gentamicin sulphate) for up to 14 days with media changes every 3 days.

Cell viability

Three separate 6 mm tissue cores were excised from both non-injured and injured discs. Two AF cores (anterior and lateral AF) and 1 NP core were excised (Fig. 1C, red circles). Viability was determined by LIVE/DEAD assay prepared in Serum-free DMEM according to manufacturer's instructions. A custom blade tool was used to prepare tissue slices for analysis as previously described (Haglund *et al.*, 2011) and samples were visualised and quantified using an inverted confocal laser scanning microscope (CLSM, Zeiss LSM 510) as previously described (Haglund *et al.*, 2011). PC12 cell viability was assessed by LIVE/DEAD assay and quantified from 3 random positions from 3 independent experiments using images obtained on an Olympus IX81 (Tokyo, Japan) inverted fluorescence microscope. All images were captured using a 20x objective with MAG Biosystems Software 7.5 (Photometrics, Tucson, AZ, USA).

Proteoglycan analysis

Sulphated glycosaminoglycans (GAGs) were quantified from culture media by a modified dimethyl methylene blue (DMMB) assay (Mort and Roughley, 2007). Samples were diluted to fall in the middle of the linear range of the standard curve and results expressed per mL medium. To quantify GAG remaining in the disc tissue post-loading, 2 AF (anterior and posterior AF) 6 mm tissue cores and 1 central NP tissue core (Fig. 1C, green circles) were extracted at 4 °C for 48 h in 15 volumes (per mg tissue wet weight) of 4 M guanidine hydrochloride (GuHCl), 50 mM sodium acetate, pH 5.8, 10 mM EDTA and protease inhibitors. In order to adjust for any interference by GuHCl, an equivalent amount of 4 M GuHCl was added to standards. Results were normalised to tissue wet weight.

Immunoblotting

For immunoblotting, only NP tissue was analysed since it normally contains the highest amount of proteoglycan in the disc. Aliquots of 8 μL of NP tissue extract (described above) were prepared for SDS-PAGE by precipitation using 9 volumes of 100 % ethanol. Precipitates were recovered by centrifugation (30 min at 4 °C). Pellets were washed once each with 75 % ethanol and 95 % ethanol before being lyophilised and re-dissolved in 25 μL 50 mM sodium acetate, pH 6.0. Resuspended protein was then digested with keratanase II at 1 μL/25 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 at 50 milliunits/25 μl of extract. Sample buffer was added directly after digestions, and the proteins were fractionated on 4-12 % SDS-PAGE gradient gels. Proteins were transferred to nitrocellulose membranes and blocked with 1.5 % (w/v) skim milk powder in TBST (0.01 M Tris-HCl, 0.15 M NaCl, 0.1 % Tween 20, pH 7.6). Antisera were diluted 1:1000 in the same buffer containing 3 % bovine serum albumin (BSA). Immunoblotting was performed using specific polyclonal antibodies that were generated against the G1 domain of aggrecan, and anti-FVDIPEN and anti-NITEGE which detect MMP and aggrecanase specific aggrecan cleavage products, respectively (Roughley *et al.*, 2012). Bound antibodies were detected by chemiluminescence using the ECL system, after incubation with a secondary antibody conjugated to horseradish peroxidise, using a LAS4000 image analyser (GE Healthcare, Baie d'Urfe, QC, Canada). ImageQuant TL software was used for pixel quantification.

Histology

Remaining disc tissue (after cores were taken) was fixed in 80 % methanol overnight at 4 °C. Additional 6 mm cores were excised (one from central NP and one from lateral AF tissue – Fig. 1C, yellow circles) from both non-injured and injured discs. Tissue cores were cryo-protected using consecutive incubations in 10 %, 20 % and 30 % sucrose solutions at 4 °C for 30 min, 1 h, and overnight, respectively and then embedded and frozen in Tissue-Tek O.C.T compound (Fisher Scientific, Whitby, ON, Canada). 15 μm cryosections were dried at 37 °C for 20 min and rehydrated with phosphate-buffered saline (PBS) for 10 min. PBS was removed and samples were stained with safranin-O and fast green as described (Purmessur *et al.*, 2013). Proteoglycan and collagen staining were visualised using a Zeiss (Oberkochen, Germany) LSM 510 META Axioplan 2 microscope and an accompanying CoolSnap (Tucson, AZ, USA) HQ CCD camera. Images

were processed using ImageProPlus6 (Media Cybernetics, Rockville, MD, USA) software.

Conditioned media analysis

Collected conditioned media from non-injured and injured disc cultures was incubated over commercially available human cytokine antibody arrays according to manufacturer's instructions. Chemiluminescence detection was performed using the ECL reagent provided in the array kit and visualised with ImageQuant LAS4000 (GE Healthcare). ImageQuant TL software was used for pixel quantification. Background baseline noise was subtracted from all samples and results expressed as a ratio of factors released from injured discs *versus* factors released from non-injured discs. The expression ratios were assessed for each disc and the average of each group was calculated. The concentration of NGF, BDNF and IL-1β in the culture medium was quantified using enzyme-linked immunosorbent assays (ELISA) according to manufacturers' instructions. Duplicate 100 µL samples of each conditioned medium were incubated in ELISA plates overnight at 4 °C. Colorimetric absorbance was measured with a Tecan Infinite M200 PRO (Tecan, Männedorf, Switzerland) and analysed with i-control 1.9 software (Tecan). Mean concentrations of each factor released by non-injured discs and injured discs were calculated.

Induction of neurite outgrowth using conditioned media

Rat pheocromocytoma, PC12 (ATCC) is a cell line that grows in semi-adherent round clusters in regular culture media. They flatten out, adhere to the plastic and differentiate towards a neuronal-like phenotype in the presence of NGF (Szeberenyi, 1996). PC12 cells also sprout axon-like projections, termed neurites, in the presence of NGF. We have previously used PC12 cells to assess the presence of neurotrophic factors released from degenerate human discs and mechanically stimulated IVD cells (Gawri *et al.*, 2014; Krock *et al.*, 2014). 2 x 10^5 PC12 cells/well were seeded on 6-well culture dishes coated with 50 mg/mL collagen type I and 0.1 % Poly-L-Lysine. Cells were allowed to attach to culture surfaces for 24 h in RPMI 1640 medium supplemented with 1 % Antibiotic-Antimycotic solution, 5 % foetal bovine serum (FBS) and 10 % horse serum. After cell attachment, control wells were supplemented with RPMI media containing 0.1 % serum (0.05 % horse serum and 0.05 % FBS) and 50 ng/mL recombinant NGF or sterile water vehicle. Remaining wells were supplemented with 1.5 mL non-injured and injured-disc conditioned media from all discs 3 days post-compression. Neurite outgrowth was monitored after 4 days. Three random phase-contrast images per sample were taken from each individual experiment (n = 3). Quantification of the number of cells with neurites was therefore averaged from 9 images (3648 x 2736 pixels each, captured using a Zeiss Axiovert 40C microscope equipped with a Canon (Tokyo, Japan) Powershot A640 digital camera attached to a Zeiss MC80DX 1.0x tube adapter) for the -NGF, NGF, non-injured and injured conditioned media samples. The whole image area was used to count cells from each condition.

Statistical analyses

All statistical analyses were performed comparing injured samples to non-injured controls using paired t-tests from three independent experiments using three individual donors. All p values less than 0.05 were considered to be statistically significant differences. Analyses were performed using Graphpad Prism 6.0 (Graphpad Software, La Jolla, CA, USA).

Results

Acute injury of healthy human IVDs fractures endplates and induces significant cell death

For non-injured controls, 5 % compression did not fracture endplates (Fig. 1A). To induce acute traumatic injury to isolated, healthy human IVDs, a single ramp compression to 30 % strain was applied at 30 % per second. This resulted in endplate fracture in all samples loaded with this protocol (Fig. 1A). The average peak stress applied to IVDs was calculated from stress-strain curves and found to be 0.212 ± 0.056 MPa for non-injured discs and 1.002 ± 0.256 MPa for injured discs (Fig. 1B). Mechanical injury of IVDs caused significant cell death (Fig. 2A). After 14 days of culture post-loading, non-injured IVDs contained about 15 % dead cells (86.16 ± 9.06 % and 81.43 ± 12.47 % cell viability in the NP and AF, respectively) (Fig. 2B). Mechanical injury resulted in about 40-50 % cell death (53.36 ± 17.91 % and 37.99 ± 15.48 % viability in the NP and AF respectively), which was significantly less than the non-injured controls (p = 0.022 and 0.017 respectively) (Fig. 2B). Data shown for AF are from lateral sections only as no differences in viability were observed between lateral and anterior AF samples (not shown).

Acute mechanical injury of healthy human IVDs causes significant loss of proteoglycan

Histological analysis of cryosections from NP and AF tissue (14 days post-loading) from non-injured IVDs showed abundant safranin-O staining, whereas NP and AF sections from injured tissue showed mostly fast-green staining (Fig. 3A). Total sulphated glycosaminoglycan (GAG) content remaining within non-injured NP tissue was 15.56 ± 6.83 mg/g while total GAG content remaining within injured NP tissue was reduced to 9.49 ± 2.64 mg/g tissue (p = 0.07) (Fig. 3B). AF tissue proteoglycan content varied greatly in both non-injured and injured discs (data not shown). Consequently, significantly more GAG (4.62 ± 0.26 mg/mL; (p = 0.0004) was detected within conditioned media from mechanically injured IVDs (3 days post-loading) compared to conditioned media from uninjured IVDs (0.11 ± 0.08 mg/mL) (Fig. 3C). GAG release to the media from the injured discs peaked at 3 days, decreased over time and reached levels comparable to the uninjured discs at 7 days post loading (data not shown).

Acute mechanical injury to healthy IVDs increases enzymatic ECM degradation

To investigate ECM integrity of the loaded IVDs, enzymatic proteoglycan degradation was assessed in NP tissue protein extracts from both non-injured and

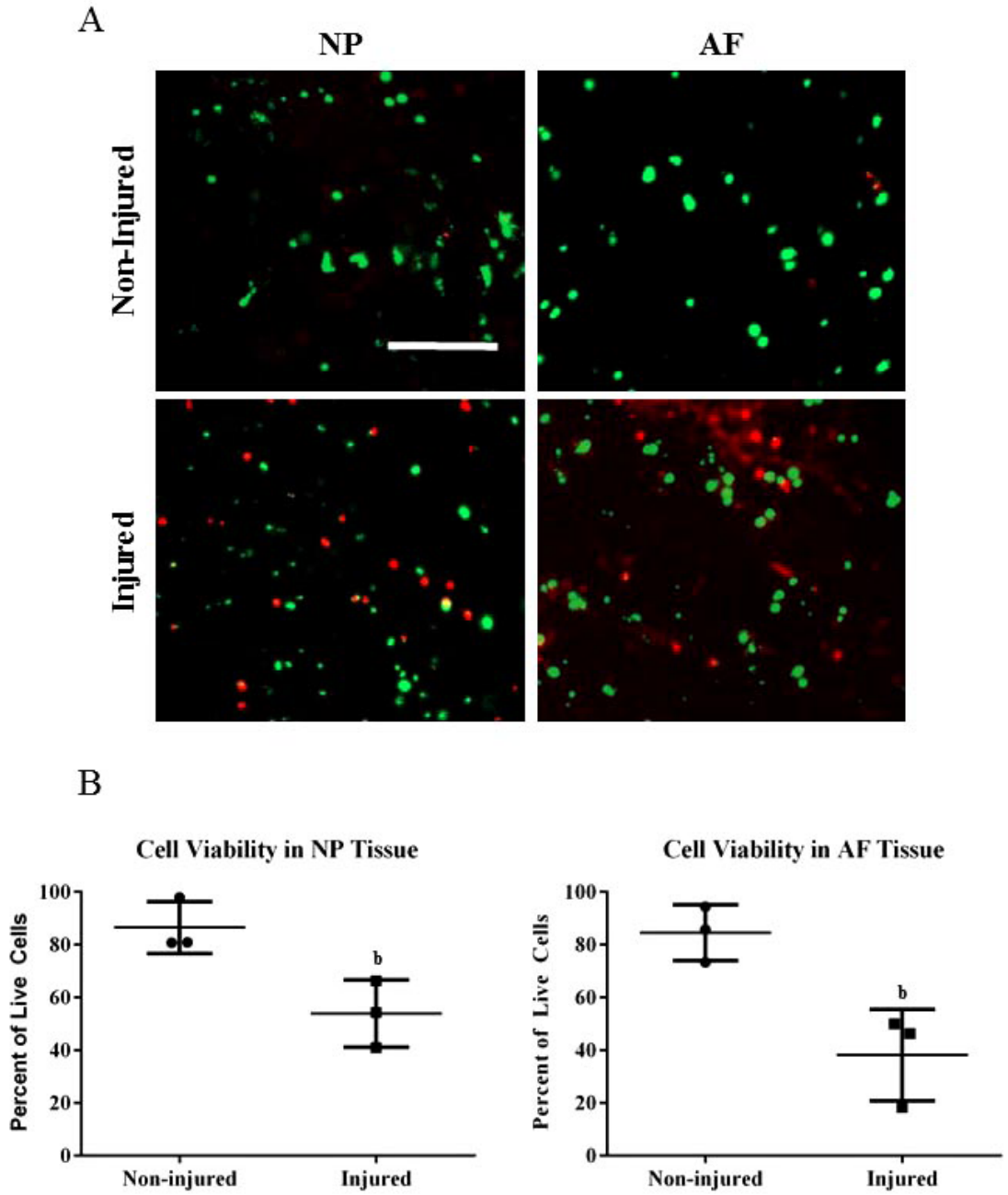

Fig. 2. Injurious mechanical loading causes significant IVD cell death. (**A**) Representative images of Live/Dead assays performed on non-injured and injured discs. Live (green) and dead (red) are shown. Cell viability was measured in 3 different tissue cores (2 AF and 1 NP) in non-injured and injured discs from three individual donors. The scale bars represent 200 μm. (**B**) The proportion of live to dead cells was quantified separately, and percentage of live cells was calculated from totals. Error bars indicate ± SD, $n = 3$. ^{b} indicates $p < 0.05$ (paired t-test).

injured discs. Western blot analysis probing for specific proteolytic sites in the core protein of aggrecan revealed increased cleavage within the interglobular domain (IGD) in tissue extracts from injured discs compared to non-injured controls (Fig. 4A). Aggrecan cleavage products of 50 kDa and 60 kDa are known to be caused by MMP and aggrecanase activity, respectively (Roughley and Mort, 2012).Therefore, to affirm that the cleavage products observed were a result of MMP and aggrecanase cleavage, tissue extracts were probed with anti-neoepitope antibodies to both the MMP and aggrecanase cleavage sites. Increased MMP and aggrecanase cleavage products were observed in tissue extracts from injured discs as compared to extracts from non-injured discs (Fig. 4A). Densitometry analysis for the 3 individual donors (3 different tissue extracts) was performed. Injured disc tissue displayed about 2 times more aggrecanase and MMP (787 ± 480 units, $p = 0.114$; 1203 ± 488 units, $p = 0.0502$) cleavage products than non-injured discs respectively (387 ± 82 units; 548 ± 68 units), albeit with some donor variation (Fig. 4B).

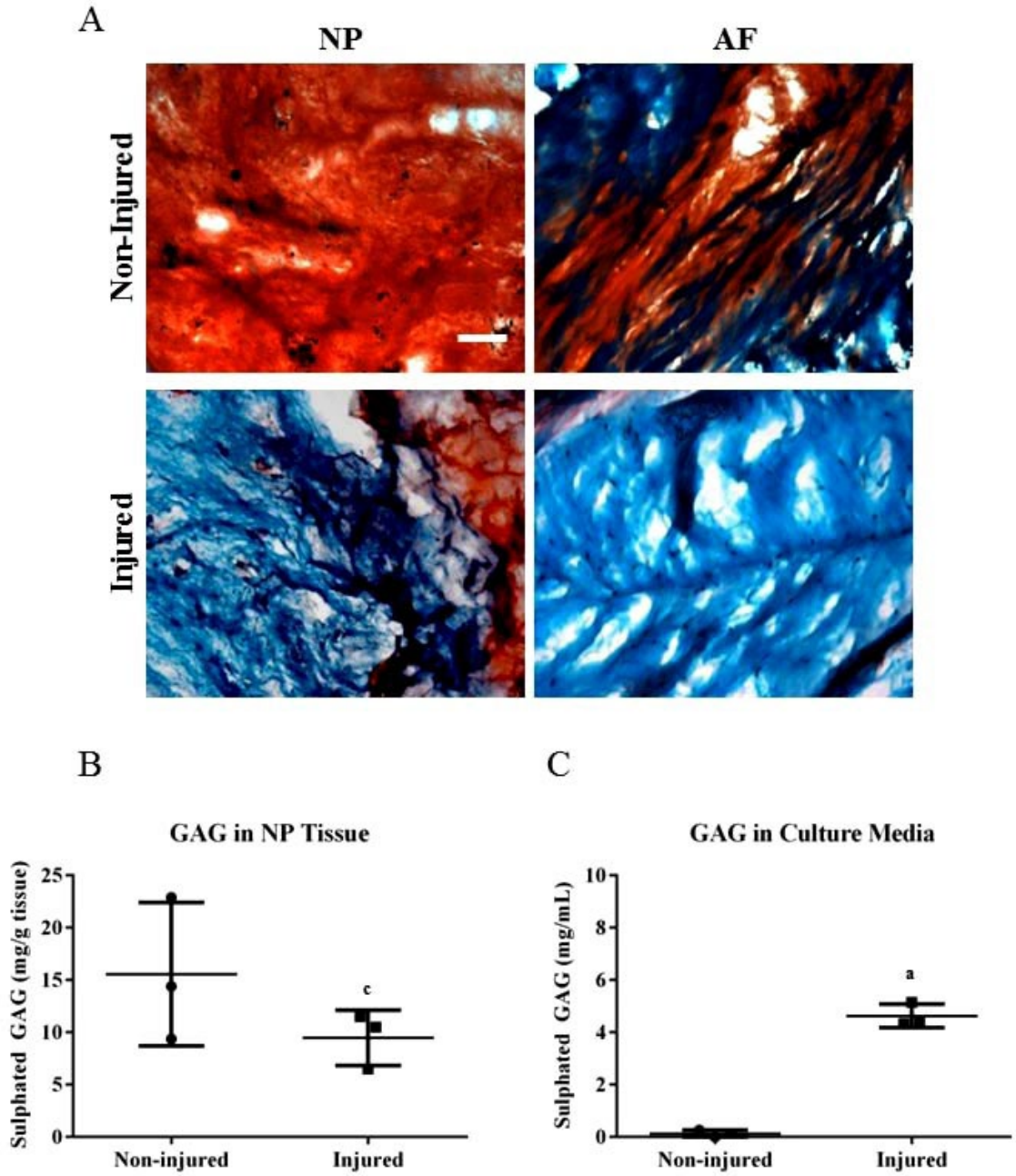

Fig. 3. Proteoglycan content within and released by injured and non-injured discs. (**A**) Representative histological sections of safranin-O/fast green staining of non-injured and injured disc tissue 14 days post-loading (scale bar, 200 μm). (**B**) Quantification of GAG content within non-injured and injured disc tissue. (**C**) Quantification of GAG released into conditioned media from non-injured and injured discs. Error bars indicate ± SD, $n = 3$. [a] indicates $p < 0.001$, [c] indicates $p = 0.07$ (paired *t*-test).

Acute mechanical injury promotes cytokine and neurotrophin release from healthy IVDs

Since inflammatory cytokines have been linked to degenerative disc disease, conditioned media from non-injured and injured discs were assessed for cytokine release. GRO-α (2.20 ± 0.68-fold; $p = 0.04$), IL-5 (1.61 ± 0.38-fold; $p = 0.032$), IL-6 (1.79 ± 0.49-fold; $p = 0.034$), IL-7 (1.65 ± 0.32-fold; $p = 0.014$), IL-8 (1.52 ± 0.31-fold; $p = 0.024$), and MCP-2 (1.22 ± 0.11-fold; $p = 0.018$) levels were significantly elevated in conditioned media from injured IVDs compared to non-injured controls (Fig. 5A). GRO (1.31 ± 0.28-fold; $p = 0.085$) and MIG (1.41 ± 0.31-fold; $p = 0.057$) showed trends for increased levels. Interestingly, IL-1α (0.79 ± 0.03-fold; $p = 0.00042$) and TGFβ-1 (0.69 ± 0.19-fold; $p = 0.031$) were significantly decreased in conditioned media from injured discs.

Since increased inflammatory responses in degenerative disc disease have been correlated with back pain, conditioned media were analysed for factors implicated in inflammatory pain, NGF, TNF-α, and BDNF (Fig. 5B).

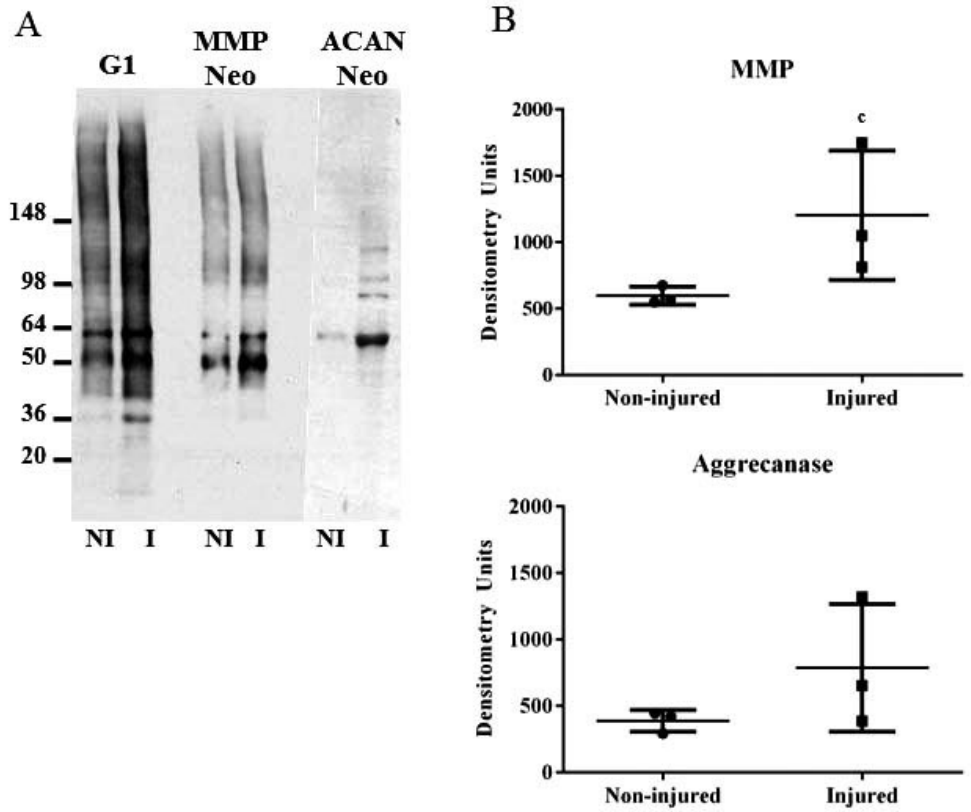

Fig. 4. Increased aggrecan degredation in injured human IVD tissue. (**A**) Representative SDS/PAGE immunoblot probed with polyclonal rabbit antibodies recognising the G1 domain of aggrecan (G1), a neo-epitope antibody recognising the MMP cleavage site of aggrecan (MMP Neo), and a neo-epitope antibody recognising the aggrecanase cleavage site of aggrecan (ACAN Neo). (**B**) Densitometry quantification of immunoblots. Error bars indicate ± SD, $n = 3$. ^{c} indicates $p = 0.0502$ (paired *t*-test).

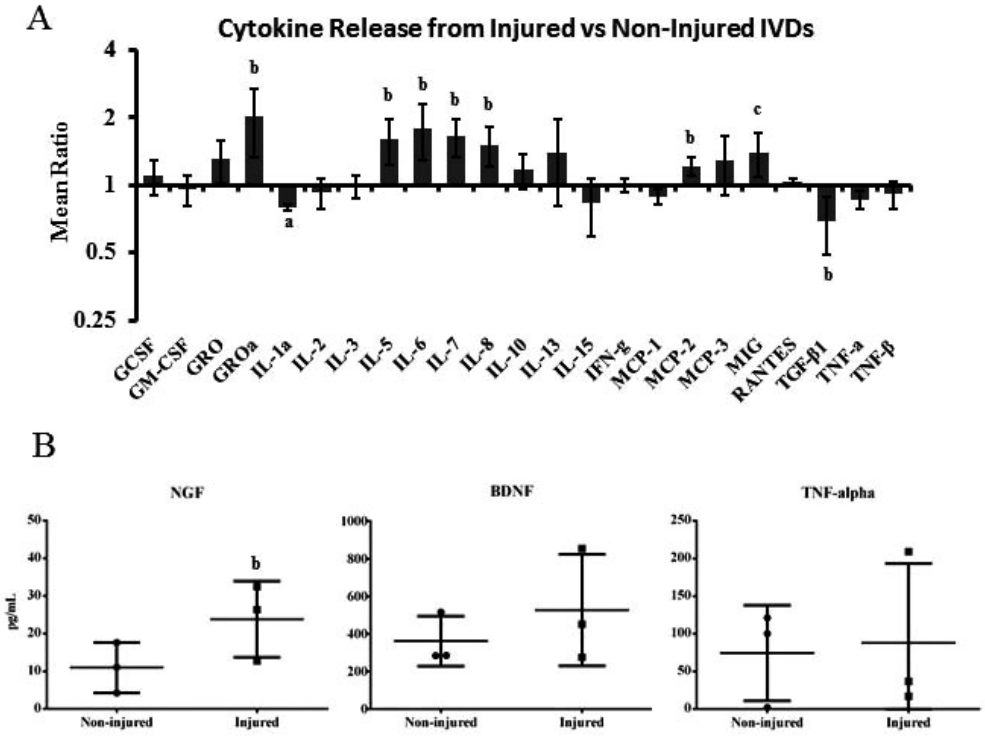

Fig. 5. Comparative analysis of factors released by injured and non-injured discs. (**A**) Media were analysed for released cytokines on a commercial array blot, and normalised to provided positive and negative controls and presented as the mean ratio. Data are plotted as factors released from injured discs *versus* non-injured discs 3 days post-loading. (**B**) ELISA analysis of conditioned culture media (3 days post-loading) from non-injured and injured discs. Error bars indicate ± SD, $n = 3$. ^{a} indicates $p < 0.01$. ^{b} indicates $p < 0.05$; ^{c} indicates $p = 0.057$, paired *t*-test.

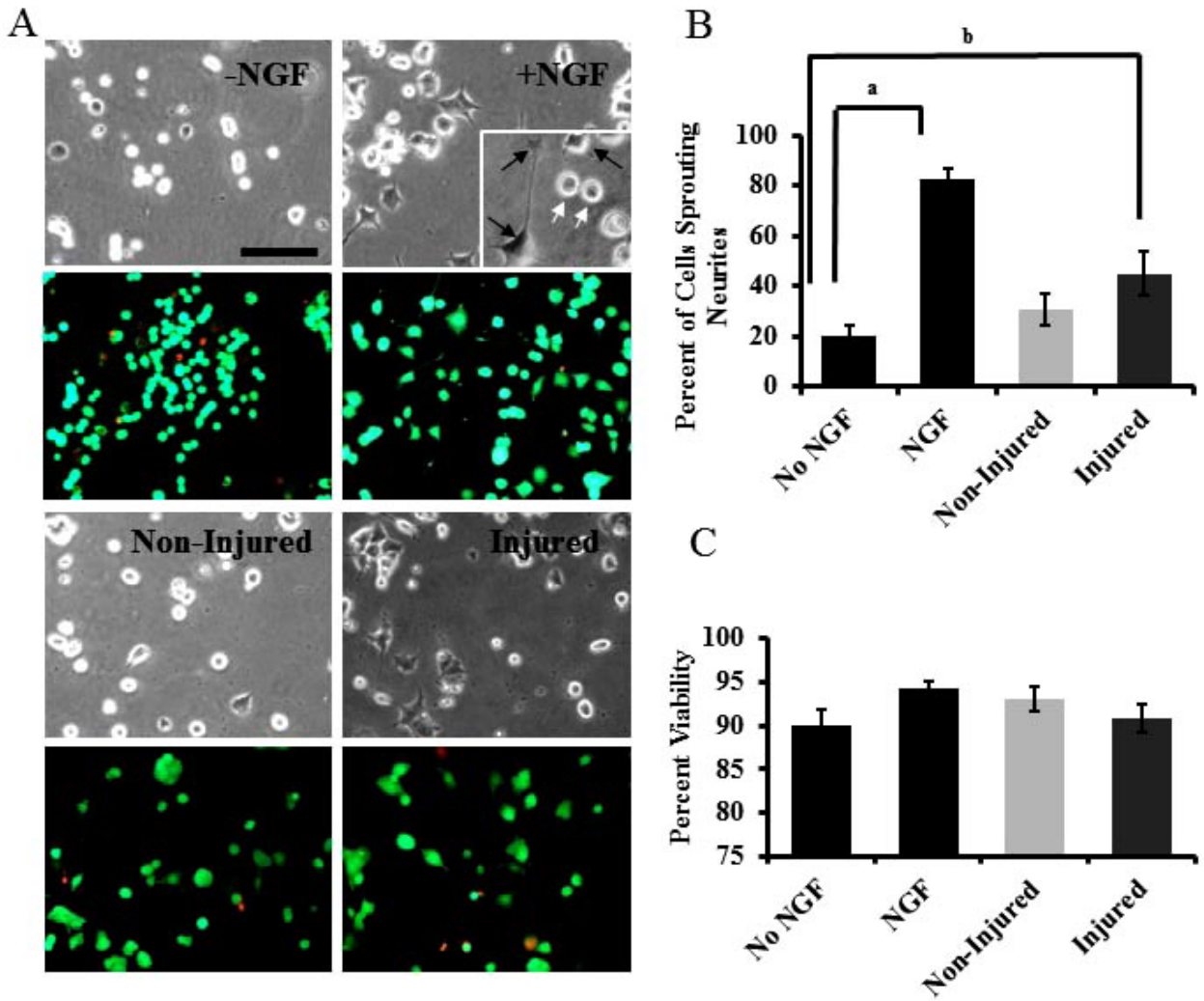

Fig. 6. Conditioned media from injured discs promote neurite outgrowth in PC12 cells. (**A**) Representative phase contrast and corresponding fluorescent LIVE/DEAD images of PC12 cells treated with sterile water vehicle (-NGF), 50 µg of NGF (+NGF), non-injured conditioned media, or injured conditioned media. Inset panel shows magnified area indicating cells with neurites (black arrows) and those without neurites (white arrows). Red and green colour indicates dead and live cells respectively. Scale bar: 100 mm. (**B**) Quantification of the proportion of cells sprouting neurites. (**C**) Quantification of cell viability. Error bars indicate ± SD. [a] and [b] indicate $p < 0.001$ and 0.05, respectively (paired *t*-test, $n = 3$ in triplicate).

NGF was significantly elevated in injured disc conditioned media (23.9 ± 10.1 pg/mL) as compared to non-injured disc conditioned media (10.9 ± 6.7 pg/mL) ($p = 0.047$) (Fig. 5B). TNF-α and BDNF levels were slightly increased in injured disc conditioned media compared to non-injured discs, however this was not statistically significant (Fig. 4B). Cytokine and neurotrophins were assessed at day 3 post-loading as preliminary data indicated that like for GAG release, their release peaked at day 3.

Conditioned media from acutely injured IVDs induces neurite outgrowth in PC12 cells

To examine whether factors released from injured IVDs can stimulate neuronal differentiation, and potentially pain, conditioned media from injured and non-injured IVDs were applied to PC12 cells and neurite outgrowth was assessed. As a positive control, addition of recombinant NGF caused robust neurite outgrowth (82.6 ± 4.2 %; $p < 0.001$) compared with untreated controls (which mainly remain rounded with no projections) (20.4 ± 4.1 %) (Fig. 6B). Neurites were visualised as axon-like projections (Fig. 6A, indicated with black arrows in the inset). Media from injured discs caused significant neurite sprouting (44.8 ± 8.9 %; $p = 0.000016$) compared to untreated controls (Fig. 6B), while conditioned media from non-injured discs caused only slightly more PC12 neurite sprouting (30.6 ± 6.3 %) than untreated controls. Since released factors such as TNFα and IL-1β may potentially induce PC12 cell death, viability was assessed (Fig. 6A). Quantification of PC12 cell viability revealed that under all conditions, viability was above 90 %, with no statistical differences (Fig. 6C).

Discussion

Mechanical injury and endplate fractures of IVDs have long been suspected to play a role in degenerative disc disease (Lotz and Ulrich, 2006; Adams *et al.*, 2009; Adams and Dolan, 2011; Chan *et al.*, 2011; Iatridis *et al.*, 2013). Here, we demonstrate that acute mechanical injury initiates events associated with disc degeneration in an *ex vivo* healthy human whole-organ IVD culture model, which lends valuable support to the current body of literature. Loading schemes of non-injurious 5 % strain (~0.2 MPa) and injurious 30 % strain (~1.0 MPa) were applied at a high speed (30 % per second), simulating normal physiological and acute hyperphysiological compressions respectively. This study shows that acute mechanical injury to healthy human IVDs causes endplate fracture, increased cell death, upregulated proteolytic enzyme activity, significant proteoglycan loss, and increased expression of inflammatory cytokines capable of stimulating neurite outgrowth. Moreover, all injured discs were compared to respective non-injured discs isolated from the same individual donors.

Previous studies using cadaveric human lumbar spines have demonstrated that complex overloading of spinal motion segments can result in vertebral endplate fracture leading to reduced intradiscal pressure (Adams *et al.*, 2000; Pollintine *et al.*, 2004) which could lead to degeneration. Acute whiplash injuries are also thought to prematurely initiate degenerative disc disease (Hamer *et al.*, 1993). Several *in vitro* models have been conducted focusing on injurious overloading of IVDs. High magnitude cyclical tensile loading of bovine caudal discs permanently disrupts the ECM (Iatridis *et al.*, 2005), and wedge-loading of bovine caudal discs causes a catabolic shift within the ECM reminiscent of degeneration (Walter *et al.*, 2011). Disc overload in an *in vivo* rat model also drives inflammation and degeneration (Miyagi *et al.*, 2012). Traumatic impact loading of rabbit IVDs caused endplate fracture, increased cell death, and upregulated mRNA expression of several inflammatory and pain markers seen in disc degeneration (Dudli *et al.*, 2012). While cyclic loading of 10-20 % strain can block catabolic activity of TNF and IL-6 in cartilage explants, dynamic strain above 30 % inhibits this effect indicating an injurious threshold at 30 % tissue strain (Li *et al.*, 2013). The present study shows that a single, fast mechanical compression of 30 % strain is sufficient to cause healthy human IVDs to immediately adapt a degenerate-like phenotype consisting of endplate fracture, proteoglycan cleavage and loss, and release of pro-inflammatory and neurotrophic factors. Taken together, our data suggests that acute trauma to IVDs may initiate events related to disc degeneration and pain *in vivo*.

IVD cell death has been previously demonstrated to be an important consequence of traumatic disc injury playing a direct role in degenerative disc disease. Using a custom drop-tower apparatus, two studies demonstrated that similar impact injuries (as applied here) can cause approximately 30-50 % cell death within injured discs (Dudli *et al.*, 2012; Dudli *et al.*, 2014). Studies have indicated that cell viability decreases in human discs as they age or become degenerate, likely contributing to matrix turnover and disc instability (Guiot *et al.*, 2000). Moreover, surgical IVD samples from patients with traumatic injury showed up to 75 % cell death (Sitte *et al.*, 2009). Here, we demonstrate traumatic impact to human IVDs causes greater than 50 % cell death in both the NP and the AF. These findings suggest that tissue necrosis plays an active role in disease progression, since the injured discs rapidly adopt a degenerate phenotype.

Proteoglycan fragmentation and loss in water content of human IVDs occur due to normal aging (Adams *et al.*, 2000), yet when these events are accelerated they are hallmarks of early disc degeneration (Song *et al.*, 2013). Proteoglycan degradation in osteoarthritic cartilage (Caterson *et al.*, 1995) or degenerate discs (Sztrolovics *et al.*, 1997) involves both aggrecanase and MMP cleavage of aggrecan serving as an indicator of matrix degradation. Moreover, proteoglycan degradation is apparent when adverse loading conditions are applied to intervertebral discs (Stokes and Iatridis, 2004; Paul *et al.*, 2013). In various animal models, this evidence is also accompanied by elevated transcript levels of matrix degrading enzymes such as MMP and ADAMTS (Maclean *et al.*, 2004; Gilbert *et al.*, 2010; Sowa *et al.*, 2011; Yurube *et al.*, 2012). In agreement with these studies, our study demonstrates significant proteoglycan cleavage and reduction in GAG content in healthy human discs subjected to acute mechanical injury as compared to non-injured discs. Previous dynamic loading studies showed increased aggrecan fragmentation (Walter *et al.*, 2011), and we demonstrate here that even a single traumatic event causes increased specific MMP and aggrecanase activity in healthy human discs thereby contributing to loss of GAG content. These data indicate that acute mechanical trauma and endplate fractures to human IVDs directly drives matrix fragmentation and reduced GAG content that could potentially initiate disc degeneration *in vivo*.

In conjunction with increased expression of catabolic proteases, previous studies have indicated that inflammatory cytokines are involved in degenerative disc disease and associated pain (Freemont *et al.*, 2002; Navone *et al.*, 2012; Krock *et al.*, 2014). An *in vivo* rat disc injury model for degeneration and pain has been established, linking increased inflammation with chronic discogenic pain (Miyagi *et al.*, 2011). Isolated IVD cells can also actively secrete these factors under various culture conditions (Yamauchi *et al.*, 2009; Moon *et al.*, 2012; Phillips *et al.*, 2013; Gawri *et al.*, 2014). In addition, exposure of IVD cells to inflammatory mediators can induce production of neurotrophic factors involved in neuronal and vascular growth (Abe *et al.*, 2007; Gruber *et al.*, 2012). Our data indicates the presence of increased cytokines and inflammatory factors post-injury. This increased inflammatory environment may be promoting the upregulated protease activity also observed post-injury (Wuertz and Haglund, 2013; Risbud and Shapiro, 2014). The present study demonstrates in real-time that high impact or adverse loading of otherwise healthy human IVDs is sufficient to induce the expression and release of inflammatory cytokines and neurotrophic factors associated with disc degeneration, neuronal and vascular infiltration and pain. Taken together, these data further

suggest that acute disc trauma can result in an inflammatory environment which may enhance disease progression and promote back pain *in vivo*.

The cartilaginous endplate model (Gawri *et al.*, 2011) we use in this study lacks several *in vivo* components of the spine, yet this *ex vivo* approach allows for direct study of the events and mechanisms causing an individual healthy human disc to acquire degenerate characteristics. Our cartilaginous endplate model allows for diffusion of nutrients and proteins into and out of the discs. Therefore conditioned culture media can be analysed for released matrix degradation products and expression of different inflammatory cytokines using western blotting, protein arrays and ELISA assays. The traumatic load applied in this study may not recapitulate exact trauma happening to individuals, it is however conceivable that very high magnitude and rapid impact resulting in endplate fractures might occur in automobile accidents, ski accidents or a fall from a height. These types of injuries could cause vertebral endplate fracture and decompression of the IVD which has been recently demonstrated (Dolan *et al.*, 2013). Furthermore, it is known now that vertebral endplate fracture can promote disc degeneration by increased expression of catabolic factors such as MMPs and pro-inflammatory factors such as TNF-α, IL-6, IL-8, MCP-1 using an *in vitro* rabbit IVD fracture model (Dudli *et al.*, 2012). It has recently been demonstrated that loaded human organ culture experiments can be performed retaining the vertebral endplates (Walter *et al.*, 2014) allowing future experiments including vertebral endplate fracture to be explored.

Conclusions

The present study shows a single hyperphysiological mechanical compression to healthy human IVDs causes endplate fracture, significant cell death, upregulated proteolytic enzyme activity, significant proteoglycan loss, and increased expression of inflammatory and neurotrophic cytokines. Interestingly, the profile of inflammatory cytokines released by the injured tissue are similar to those released by surgical samples removed from patients suffering painful disc degeneration. Factors released by the injured tissue are also known neurotrophins, capable of promoting neuronal growth and sensitisation. These data indicate that the acute mechanical injury of healthy human IVDs can promote early events associated with disc degeneration, inflammation and potential pain. Moreover, data obtained from these studies may provide new insights to better diagnostic and therapeutic approaches to spine injury, regenerative medicine and pain treatment. Future studies will focus on injury of tissue with vertebrae intact, to better model traumatic IVD injury as it occurs *in vivo*.

Acknowledgements

This work was supported by the Canadian Institutes of Health Research (CIHR) grant CIHR MOP-119564 and AOSpine , SRN_2011_04_10498. The authors would like extend thanks to Rahul Gawri for his role in harvesting IVDs from human organ donors. BA and DHR designed the study, conducted experiments, analysed data, interpreted results and wrote the manuscript. EK and LB contributed to experiments. MHW, JAO, TS and PJR helped to design the study and reviewed and revised manuscript. MHW and JAO provided organ donor IVD samples. LH designed the study, interpreted results, and wrote the manuscript. All authors approved of the final manuscript.

Disclosures: All authors disclose that there are no conflicts of interest.

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Discussion with Reviewers

S. Grad: Removal of vertebral endplates and rapid loading are artificial conditions used in this model system. How do the cartilage endplate injuries observed in this study compare with endplate defects observed on human histology, or Modic changes observed on MRI in clinical patients?

Authors: It is difficult to make a direct correlation to the *in vivo* situation, however, experimental rapid loading causing fracture to the cartilage endplate most likely mimics an acute injury. We performed preliminary experiments where the cartilage endplate was cut open without a mechanical injury and could not see an increase in proteinases, cytokines or pain mediators, whereas non-aggregating proteoglycan rapidly diffused from the tissue into the culture medium. Based on this one would speculate that endplate fracture, Modic changes or any defect or increased

permeability of the cartilage endplate would facilitate proteoglycan loss, which with time would lead to altered tissue properties and perhaps non-physiologic stress on the cells, that in turn could result in increased proteinase, cytokine and pain mediator production.

J. Iatridis: It is interesting to note that the release of cytokines appeared to peak at day 3 after injury. This suggests a natural reaction to acute tissue damage that is generally regarded as beneficial for inducing a repair response. In contrast, a sustained inflammatory state has been associated with progressive degenerative diseases. Does the outcome suggest an entirely catabolic response, or is a certain anabolic reaction likely at later time points?
Authors: The reviewer raises and interesting and valid point. It is indeed true that acute inflammatory responses after injury are involved in tissue repair processes, while chronic or prolonged inflammatory environment can play a direct role in disease progression. Our experiments are conducted in an open *ex vivo* culture system where inflammatory factors are released into the culture medium. We replenish culture media every 3 days with the consequence that the released inflammatory factors are being removed or at least diluted – making it difficult to fully address the question. Nonetheless, the observation that enzymatic matrix degradation is occurring also at 14 days suggests a sustained catabolic environment. Had the study been conducted *in vivo* in a "closed system", we suspect that the elevated levels of inflammatory factors and proteinases could possibly generate matrix fragments activating Toll-like receptors and would remain within the tissue driving stronger and more sustained responses. Only further studies on acute disc trauma in a large animal model would be able to fully address whether or not the response is sustained and results in degeneration.