Re-Engineering Lumbar Microdiscectomy:

From the Bench to the Bedside

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MBBS MPhil. BSc.

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Abstract

Lumbar microdiscectomy is the most commonly performed spine surgery worldwide. The procedure is successful in relieving radicular symptoms, however, fails to address the underlying pathophysiology of disc degeneration. Consequently, up to one third of patients progress to suffer chronic low back pain, recurrent disc herniation occurs in up to 15% and reoperation may be required in up to 25% of this cohort. Furthermore, post-operative care protocols following lumbar discectomy remain markedly heterogeneous reflecting the lack of evidence regarding the role, if any, of post-operative activity restrictions following lumbar discectomy.

The aim of this thesis was to re-engineer the lumbar microdiscectomy procedure via pre-clinical and clinical studies. The preclinical studies utilized an ovine lumbar intervertebral disc injury model due to the anatomical, biomechanical, biochemical and cellular similarities to the human intervertebral disc. The first preclinical study compared a modified annulotomy injury model with a drill bit injury model with the aim of developing an appropriate large animal model of the post lumbar discectomy intervertebral disc. This study demonstrated that after six months the modified annulotomy injury produced more advanced intervertebral disc degeneration than the drill bit injury as assessed by radiological, gross morphological, biochemical and histological analysis and a defect more consistent with that observed following clinical lumbar discectomy. As such the modified annulotomy model was used to investigate novel cellular therapies in the subsequent component.

Next, this thesis investigated a novel cellular therapy for intervertebral disc degeneration to be administered at the time of lumbar microdiscectomy. This therapy consisted of allogeneic mesenchymal progenitor cells (MPCs), the earliest uncommitted clonogenic population of bone marrow stromal cells, primed with pentosan polysulfate(PPS). PPS priming of MPCs in culture for 24-48hrs has been demonstrated to promote MPC proliferation and chondrogenic differentiation that persisted following removal of MPCs from the PPS in vitro culture. Priming potentially offer regulatory advantages over PPS and MPC co-administration. This therapy was investigated in the modified annulotomy model. Sheep were allocated into three groups: the injury group received no further treatment, the pMPC group received 0.5×10^6 PPS primed MPCs in a gelatin matrix with fibrin sealant and the MPC group received 0.5×10^6 unprimed MPCs in the same matrix with fibrin sealant. At six months the pMPC discs demonstrated superior regeneration on gross morphological, biochemical and histological analysis compared to both the MPC and injury only discs with markedly less vascular and cellular infiltration.
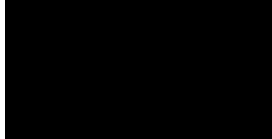
The clinical effort to optimize lumbar microdiscectomy commenced with a survey investigating the perioperative practices of Australasian neurosurgeons. This survey demonstrated heterogeneity in post-

operative care practices consistent with the international literature. In order to investigate the role, if any, of post-operative activity restrictions following lumbar microdiscectomy, a single blinded multi-centre randomised controlled trial was designed and commenced. On interim analysis 89 patients had undergone randomisation and lumbar microdiscectomy with five patients suffering MRI confirmed intervertebral disc reherniation. Three of these patients were in the activity restrictions group with the remaining two patients in the unrestricted group. Recruitment is anticipated to complete in 2020.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature:

Print Name: Dr Chris Daly

Date: 23/01/2018

Publications, Presentations, Grants, Book Chapters, Prizes and Clinical Trials Arising From This Thesis

Published Journal Articles

1. Daly C, Ghosh P, Jenkin G, Oehme D, Goldschlager T. A Review of Animal Models of Intervertebral Disc Degeneration: Pathophysiology, Regeneration, and Translation to the Clinic. Biomed Res Int. 2016;2016(3):5952165–14.

2. Lim K-Z, Daly CD, Ghosh P, Jenkin G, Oehme D, Cooper-White J, et al. Ovine Lumbar Intervertebral Disc Degeneration Model Utilizing a Lateral Retroperitoneal Drill Bit Injury. J Vis Exp. 2017 May 25;(123).

3. Daly CD, Ghosh P, Zannettino ACW, Badal T, Shimmon R, Jenkin G, et al. Mesenchymal progenitor cells primed with pentosan polysulfate promote lumbar intervertebral disc regeneration in an ovine model of microdiscectomy. Spine J. 2017 Oct 18.

4. Daly CD, Lim K-Z, Ghosh P, Goldschlager T. Perioperative care following lumbar microdiscectomy a survey of Australasian Neurosurgeons. J Spine Surg. Accepted October 2017.

5. Daly CD, Lim K-Z, Lewis J, Saber K, Molla M, Bar-Zeev N, et al. Lumbar microdiscectomy and post-operative activity restrictions: a protocol for a single blinded randomised controlled trial. BMC Musculoskelet Disord. 2017 Jul 20;18(1):312.

6. Wu J, Shimmon S, Paton S, Daly C, Goldschlager T, Gronthos S, et al. Pentosan polysulfate binds to STRO-1+ mesenchymal progenitor cells, is internalized, and modifies gene expression: a novel approach of pre-programing stem cells for therapeutic application requiring their chondrogenesis. Stem Cell Res Ther. 2017 Dec 13;8(1):278.

Submitted Journal Articles (Currently Under Review)

1. Daly CD, Ghosh P, Badal T, Shimmon R, Jenkin G, Oehme D, Cooper-White J, Sher I, Chandra RV, Goldschlager T. A Comparison of Two Ovine Lumbar Intervertebral Disc Injury Models for the Evaluation and Development of Novel Regenerative Therapies. Submitted to Global Spine Journal.

2. Daly CD, Ghosh P, Goldschlager T. Back pain, the opioid crisis and novel alternatives. Submitted to ANZ J Surg.

Abstracts

1. Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Sher I, Vais A, Cohen C, Goldschlager T. Pentosan polysulfate primed mesenchymal progenitor cells promote intervertebral disc repair following microdiscectomy in an ovine model. Presented at the Monash Health Translation Precinct Research Week, November 2017, Melbourne, Australia. (Oral Presentation)

2. Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Sher I, Vais A, Cohen C, Goldschlager T. Pentosan polysulfate primed mesenchymal progenitor cells promote intervertebral disc repair following microdiscectomy in an ovine model. Presented at the 54th Annual Scientific Meeting of the Surgical Research Society of Australasia. November 2017, Adelaide, Australia (Oral Presentation)

3. Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Sher I, Vais A, Cohen C, Goldschlager T. Pentosan polysulfate primed mesenchymal progenitor cells promote intervertebral disc repair following microdiscectomy in an ovine model. Presented at the North American Spine Society 32nd Annual Meeting, October 2017, Orlando USA. (Oral Presentation)

4. Sher I, Oehme D, Daly CD, Chandra RV, Sher M, Fulcher A, Cohen C, Vais A, Smith JA, Goldschlager T. Histological, Radiological, Immuno-Histochemical and Birefringence Investigation of the Mechanisms of Action of Mesenchymal Progenitor Stem Cells (MPCs) in the Early Repair of Degenerative Disc Disease Using the Ovine Annulotomy Injury Model. Presented at the North American Spine Society 32nd Annual Meeting, October 2017, Orlando USA. (Oral Presentation)

5. Sher I, Daly CD, Oehme D, Chandra RV, Ghosh P, Sher M, Smith JA, Goldschlager T. Could the Transitional Zone be the Key to Predicting Degenerative Disc Disease? Presented at the North American Spine Society 32nd Annual Meeting, October 2017, Orlando USA. (Poster Presentation)

6. Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Sher I, Vais A, Cohen C, Goldschlager T. Intervertebral disc repair following microdiscectomy mediated by pentosan polysulfate primed mesenchymal progenitor cells in an ovine model. Presented at the Congress of Neurological Surgeons 2017 Annual Meeting, October 2017, Boston USA. (Oral Presentation)

7.   Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Sher I, Vais A, Cohen C, Goldschlager T. Pentosan polysulfate primed mesenchymal progenitor cells promote intervertebral disc repair following microdiscectomy in an ovine model. Presented at the Neurosurgical Society of Australasia Annual Scientific Meeting 2017 Adelaide, Australia. (Oral Presentation)

8.   Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Sher I, Vais A, Cohen C, Goldschlager T. Mesenchymal progenitor cells primed with pentosan polysulfate mediate disc repair following microdiscectomy in an ovine model. Presented at the Global Spine Congress, May 2017, Milan. (Oral Presentation)

9.   Sher I, Daly C, Goldschlager T, Oehme D, Chandra R, Ghosh P. 9.4T MRI complements the Pfirrmann grade through better differentiation of the NP/AF. Presented at the Global Spine Congress, May 2017, Milan. (Oral Presentation)

10.   Daly CD, Ghosh P, Badal T, Shimmon R, Ghosh I, Jenkin G, Oehme D, Cooper-White J, Naidoo T, Sher I, Jain K, Oehme D, Goldschlager T. Radiological, Histological, Morphological and Biochemical Comparison of two Ovine Lumbar Intervertebral Disc Injury Models. Presented at the Global Spine Congress, May 2017, Milan. (Oral Presentation)

11.   Daly CD, Lim KZ, Ghosh P, Lewis J, Saber K, Buchanan M, Goldschlager T. Post-operative care following lumbar microdiscectomy: a survey of Australasian Neurosurgeons. Presented at the Global Spine Congress 2017, Milan. (Poster)

12.   Sher I, Sher M, Daly C, Fulcher A, Oehme D, Ghosh P, Smith J, Goldschlager T. Detailed analysis of intervertebral disc structure using the 9.4T MRI and polarized light microscopy facilitates understanding of disc biomechanics. Presented at the Spine Society of Australia Annual Scientific Meeting 2017, Hobart. (Oral Presentation)

13.   Oehme D, Ghosh P, Goldschlager T, McDonald C, Daly C, Troupis J, Rosenfeld JV, Jenkin G. Disc Regeneration Using STRO-3$^+$ Immunoselected Allogeneic Mesenchymal Precursor Cells Combined with Pentosan Polysulfate. NSA Sydney Oct 2016. (Oral Presentation)

14.   Daly C, Goldschlager T, Ghosh P, Jenkin G, Cooper-White J, Naidoo T. Radiological, Histological and Biochemical Characterization of a Novel Ovine Lumbar Intervertebral Disc Injury Model. Presented at the World Forum for Spine Research Dubai 2016. (Oral Presentation)

15.   Daly C, Oehme D, Ghosh P, Goldschlager, Rosenfeld J, Troupis J, McDonald C, Jenkin G. Mesenchymal progenitor cells combined with pentosan polysulfate can mediate disc regeneration at the time of microdiscectomy. Presented at the XIIth annual Adelaide Centre for Spinal Research Symposium Adelaide 2014. (Oral Presentation)

Book Chapters

1. Daly, C and Goldschlager, T. *Chapter 24: Surgical versus Nonsurgical Therapy for Lumbar Spinal Stenosis* in 50 Landmark Papers Every Spine Surgeons Should Know. In press.

Grants

1. 2016 Cabrini Foundation Research Grant ($30,000) for "Lumbar microdiscectomy and post-operative activity restrictions: a protocol for a single blinded randomised controlled trial"

Prizes

1. 2015-2017 Royal Australasian College of Surgeons Foundation for Surgery Richard Jepson Research Scholarship ($180,000).

2. 2016 Monash Institute of Medical Engineering Funded Scholarship to attend SPARK Bioinnovation and Entrepreneurship Course at Stanford 2016 ($5000).

3. 2017 Surgical Research Society of Australasia Developing Careers in Academic Surgery Grant. Awarded for best oral presentation at Surgical Research Society of Australasia annual meeting Adelaide Australia ($750).

Clinical Trials

1. Impact of post-operative activity restriction on lumbar microdiscectomy clinical outcome: a single blinded randomised controlled trial. *Australian New Zealand Clinical Trials Registry (ANZCTR), World Health Organization Primary Registry. Approval number:* ACTRN12616001360404

2. A Prospective, Multicenter, Randomised, Double-blind, Placebo-controlled Study to Evaluate the Efficacy and Safety of a Single Injection of Rexlemestrocel-L or Combined With Hyaluronic Acid (HA) in Subjects with Chronic Low Back Pain. *ClinicalTrials.gov Identifier NCT02412735*

Thesis Including Published Works Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes six original papers published or in press in peer reviewed journals and two manuscripts submitted for review. The core theme of the thesis is the re-engineering of lumbar microdiscectomy surgery. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Surgery under the supervision of Assoc. Prof. Tony Goldschlager, Prof. Peter Ghosh and Prof. Graham Jenkin.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)
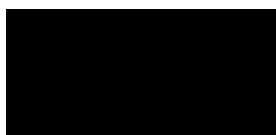
In the case of *(Chapters 1, 2, 3, 4, 5, and 6 and Appendix 3)* my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status (published, in press, accepted or returned for revision, submitted)	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
1	A Review of Animal Models of Intervertebral Disc Degeneration: Pathophysiology, Regeneration, and Translation to the Clinic	Published	Concept, design, writing and revising manuscript. 80%.	1. Peter Ghosh, writing and revising manuscript, 5%	N
				2. Graham Jenkin, revising manuscript, 5%	N
				3. David Oehme, revising manuscript, 5%	N
				4. Tony Goldschlager, concept, writing and revising manuscript, 5%	N

2	Ovine Lumbar Intervertebral Disc Degeneration Model Utilizing a Lateral Retroperitoneal Drill Bit Injury	Published	Concept, writing and revising draft, acting in accompanying video. 55%.	1. Kai-Zheong Lim, writing and revising manuscript 30%	N
				2. Peter Ghosh, concept 5%	N
				3. Graham Jenkin, concept 2%	N
				4. David Oehme, concept, writing and revising manuscript 1%	N
				5. Justin Cooper-White, concept 1%	N
				6. Taryn Naidoo, concept 1%	N
				7. Tony Goldschlager, concept, revising manuscript, 5%	N

3	A Comparison of Two Ovine Lumbar Intervertebral Disc Injury Models for the Evaluation and Development of Novel Regenerative Therapies	Submitted	Concept, design, conduct and interpretation of experiments, writing and revising manuscript. 80%.	1. Peter Ghosh, concept, design of experiments, revising manuscript 5%	N
				2. Tanya Badal, biochemistry 2%	N
				3. Ronald Shimmon, biochemistry 2%	N
				4. Graham Jenkin, concept 2%	N
				5. David Oehme, concept, writing and revising manuscript 1%	N
				6. Justin Cooper-White, concept 1%	N
				7. Idrees Sher, experiments 1%	Y
				8. Ronil Chandra, experiments 1%	N
				9. Tony Goldschlager, concept, revising manuscript, 5%	

4	Mesenchymal progenitor cells primed with pentosan polysulfate promote lumbar intervertebral disc regeneration in an ovine model of microdiscectomy	Published	Concept, design, conduct and interpretation of experiments, writing and revising manuscript. 80%.	1. Peter Ghosh, concept, design of experiments, revising manuscript 5%	N
				2. Andrew Zannettino, concept 1%	N
				3. Tanya Badal, experiment performance 1%	N
				4. Ronald Shimmon, experiment performance 1%	N
				5. Graham Jenkin, concept 1%	N
				6. David Oehme, concept, writing and revising manuscript 1%	N
				7. Kanika Jain, experiment performance 1%	N
				8. Idrees Sher, experiments 1%	Y
				9. Angela Vais, experiment performance 1%	N
				10. Camilla Cohen, experiment performance 1%	N
				11. Ronil Chandra, data interpretation 1%	N
				12. Tony Goldschlager, concept, revising manuscript, 5%	N

5	Perioperative care for lumbar microdiscectomy: a survey of Australasian Neurosurgeons	Accepted	Concept, design, conduct and interpretation of survey. Writing and revising manuscript. 80%.	1. Kai-Zheong Lim, conduct of experiment, 5%	N
				2. Peter Ghosh, writing and revising manuscript, 5%	N
				3. Tony Goldschlager, concept, writing and revising manuscript, 10%	N
6	Lumbar microdiscectomy and post-operative activity restrictions: a protocol for a single blinded randomised controlled trial	Published	Concept, design, writing and revising manuscript. 80%.	1. Kai Zheong Lim, concept, 2%	N
				2. Jennifer Lewis, concept 2%	N
				3. Kelly Saber, concept 2%	N
				4. Mohammed Molla, writing manuscript 2%	N
				5. Naor Bar-Zeev, writing and revising manuscript, 2%	N
				6. Tony Goldschlager, concept, writing and revising manuscript 10%	N
Appendix 3	Back pain, the opioid crisis and novel alternatives	Submitted	Concept, design, writing and revising manuscript. 80%.	1. Peter Ghosh, revising manuscript 5%	N
				2. Tony Goldschlager, concept, revising manuscript, 15%	N

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.
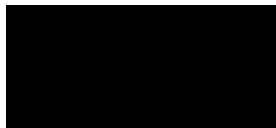
Student signature:

Date: 23/01/2018

Main Supervisor signature:

Date: 23/01/2018

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I would like to acknowledge and thank my supervisors Assoc. Prof. Tony Goldschlager, Prof. Peter Ghosh and Prof. Graham Jenkin. I would particularly like to thank Assoc. Prof Tony Goldschlager and Prof. Peter Ghosh for their generosity in time and guidance over the previous three years. Additional thanks go to Dr David Oehme for his help and guidance. Thanks also go to Mrs Jennifer Lewis, Ms Jessica Brown, Ms Hema Navaratnam, Ms Sophie Jennings and Ms Kylie Fryer for their assistance with the clinical trials. Thank you to all at the Ritchie Centre, Hudson Institute of Medical Research, Monash University Department of Surgery, Monash Animal Services, Monash Biomedical Imaging, Monash Histology Platform, Proteobioactives, University of Technology Sydney and the laboratories of Professor Andrew Zannettino at the University of Adelaide and Professor Justin Cooper-White at the University of Queensland. Their work has made this PhD possible.

I would also like to personally acknowledge the following people for their help and assistance: Dr Anne Gibbon, Dr Dong Zhang, Dr Kai-Zheong "Teddy" Lim, Dr Reece Sher, Dr Yen Pham, Assoc. Prof. Ronil Chandra, Prof. Andrew Zannettino, Dr Kanika Jain, Dr Ruth Vreys, Dr James Pearson, Dr Qizhu Wu, Ms Camilla Cohen, Ms Angela Vais, Ms Tanya Badal, Dr Ronald Shimmon, Mr Ian Ghosh, Mr Myron Rogers, Mr Andrew Danks and Prof. Julian Smith. I would also like to thank all the doctors and neurosurgeons who have taught and mentored me over the years, and who have supported me over the PhD journey.

I would like to give special thanks to my family and Sarah for their ongoing support.

Dedication

This thesis is dedicated to my parents, Andrew and Karen Daly.

Chapter 1.   Introduction

This chapter consists of a literature review and introduction to the thesis and includes sections of a manuscript published in BioMed Research International entitled, **"A Review of Animal Models of Intervertebral Disc Degeneration: Pathophysiology, Regeneration, and Translation to the Clinic"** rewritten into the format of a thesis chapter. The manuscript is included at the end of this chapter.

The candidate, Chris Daly, contributed to the design, writing and revision of the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xv.

1.1. Introduction

Low back pain is the leading cause of disability worldwide (1,2), and is strongly associated with disc degeneration. Disc degeneration arises from multifactorial etiologies that include ageing, physical activity, microtrauma, smoking, obesity, hormonal dysregulation and genetics(3). The degenerative process often commences in the nucleus pulposus, the gelatinous core of the disc, and extends to the annulus fibrosus (AF), the fibrous outer layer. When coupled with mechanical loading, this may lead to annular failure, disc herniation and subsequent compression of the neural elements producing radicular pain or radiculopathy. Failure of conservative management of symptomatic herniation with neural compression may require surgical treatment with lumbar microdiscectomy.

Lumbar microdiscectomy is the most common spinal operation performed globally with over 300,000 such procedures undertaken annually in the United States alone(4). Unfortunately, lumbar microdiscectomy, while relieving the radicular symptoms, fails to address the underlying pathophysiology of disc degeneration that contributed to the annular failure. Consequently, up to one third of patients progress to suffer chronic low back pain(5) with this being disabling in approximately 10%(6). Recurrent disc herniation occurs in 5-15% of patients and reoperation may be required in 4-25% of this cohort(7). Approximately 40% of these patients will subsequently progress to fusion surgery within four years(8). This is major surgery, where the entire disc is removed and the adjacent vertebral bodies fused to each other.

Lumbar fusion surgery, however, is not a panacea for discogenic back pain, with many patients failing to demonstrate a significant improvement in lower back pain and many undergoing additional spine surgery in the future(9). As such lumbar fusion surgery for discogenic back pain remains controversial(10). Given the global disease burden of back pain and the failure of conventional therapies to address the underlying pathobiology of disc degeneration, concerted efforts to develop alternative modalities of treatment are currently being made throughout the world. Mesenchymal stem cells (MSC) have long been used in spinal surgery, originally in the form of iliac bone marrow explants, to promote bone fusion, but more recently in their purified form where they have been evaluated as potential candidates to induce disc repair (11). Our group have demonstrated, that when mesenchymal progenitor cells (MPCs), a subpopulation of MSCs, are combined with the pharmaceutical agent pentosan polysulfate (PPS), and administered in an ovine model of disc degeneration intervertebral disc (IVD) regeneration is promoted, as assessed by reductions in disc height index loss, improved gross morphology, histological appearance, and improved disc biochemistry(11,12). Furthermore, this MPC and PPS combination has demonstrated the ability to promote intervertebral disc regeneration in an ovine model of the post lumbar discectomy intervertebral disc in a pilot study(11). Thus, there remains significant opportunity to optimize the lumbar discectomy procedure by administration of

regenerative therapies at the time of surgery. This obviates the need for additional metachronous procedures.

Furthermore, despite lumbar discectomy being the most commonly performed spine surgery procedure in the world, significant heterogeneity still exists in post-operative care practices following the procedure, with patients on the same ward often being subjected to markedly different post-operative activity protocols, depending on their surgeon(13). This heterogeneity is reflective of the lack of evidence regarding the role, if any, of post-operative activity restrictions following lumbar discectomy.

The focus of this thesis is to re-engineer the lumbar discectomy procedure. Optimization of two aspects of this procedure will be explored. First, this thesis will describe the development of a novel cellular therapy to be administered at the time of lumbar discectomy surgery to promote intervertebral disc regeneration. If this is successful, patients will not only enjoy alleviation of radicular (leg pain) but also a reduction in back pain and a reduction in recurrent disc herniation and further surgery. Second, this thesis will investigate the role of post-operative activity restrictions following lumbar discectomy, with an aim to provide clear evidence for patients and clinicians on optimal care. In order to provide relevant background it is important to first address the relevant anatomical and pathophysiological considerations and discuss contemporary management of lumbar disc degeneration.

1.2. Lumbar Spine Anatomy

1.2.1. Human Lumbar Spine Anatomy

The human vertebral column consists of five regions with characteristic anatomy: cervical, thoracic, lumbar, sacral and coccygeal. The lumbar spine consists of five vertebrae, which articulate cranially with the thoracic spine and caudally with the sacrum. The lumbar spine possesses a lordosis, an anterior convexity, much of which is provided by the intervertebral discs.

The typical lumbar vertebra consists of an anterior vertebral body and posterior vertebral (neural) arch. The vertebral foramen is enclosed between the anterior vertebral body and posterior vertebral arch and encases the spinal cord until its termination at approximately the L2 level in humans. The neural arch consists of the pedicle (between the vertebral body and transverse process) and the lamina, posterior to the transverse process. The intervertebral foramina for exiting spinal nerve roots lie between the pedicles of adjacent vertebra. The vertebrae articulate posteriorly at the facet joints and are separated anteriorly by intervening intervertebral discs.

1.3. The Intervertebral Disc

The intervertebral disc is a complex multi-component structural tissue consisting of an outer fibrous ring, the annulus fibrosus (AF), and an inner hydrated gel-like substance, the nucleus pulposus (NP)(14) (see Figure 1). It is the largest avascular structure in the body. Nutrition of the intervertebral disc is provided by diffusion through the cartilaginous endplates (CEP). The CEP are specialized interfaces that connect the intervertebral disc with the adjacent vertebral bodies. The AF is a fibrocartilagenous tissue rich in type I and II collagens and assembled as lamellae fibres oriented at varying degrees to adjacent lamella in different locations and species. The AF connects the caudal and cranial vertebral bodies of the spinal column (15). The main cell types of the AF are fibroblasts that not only synthesize the lamellar collagens, but also proteoglycan (PGs), elastin and other non-collagenous proteins(16). The tough fibrous composite structure of the AF encapsulates the gelatinous NP and provides the necessary mechanical strength and resilience to allow the disc to recover from deformation arising from axial, rotational and bending loading. In healthy discs the NP consists of a hydrated gel composed of predominantly type II collagen and large amounts of PGs. Aggrecan is the most abundant PG type in the NP that, because of it high anionic charge, attracts and retains high levels of water molecules within the NP thereby maintaining a high hydrostatic swelling pressure that confers resistance to disc deformation and maintenance of disc height(14,17).

Cells of the disc NP are derived from the notochord. In man these cells are retained throughout childhood but with maturity disappear and are replaced by chondrocyte-like cells(16). The loss of notochordal cells from the NP represents an important early step along the path to degenerative disc disease.

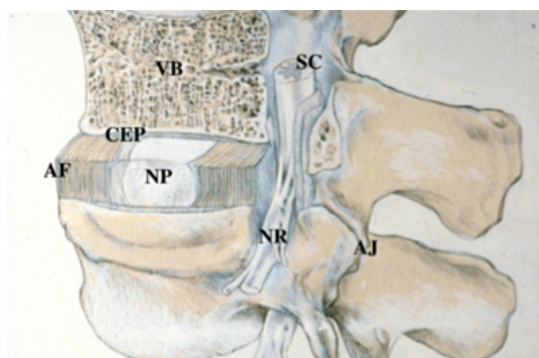**Figure 1.** Schematic view of the intervertebral disc and spinal segment from Urban et al.(18). The figure shows the outer lamellated annulus fibrosus (AF), inner gelatinous nucleus pulposus and the cartilagenous endplates (CEP)- the specialized interface between the intervertebral disc and vertebral body (VB). The figure also shows the related nerve root (NR), spinal cord (SC) and apophyseal joint (AJ).

1.4. Aetiology and Pathophysiology of Intervertebral Disc Degeneration

Human intervertebral disc degeneration is a complex and incompletely understood multifactorial process with contributions from genes, mechanical stresses, cellular senescence, and alterations in nutrition via the limited vascular supply(3).

Ultimately there is an imbalance between the rates of production and breakdown of the matrix components, reduction in cellular viability and activity and alterations in cytokine profile upregulating the breakdown of proteoglycans- leading to dehydration and loss of mechanical integrity of the intervertebral disc(17,18).

The degeneration of the annulus fibrosus and subsequent tears in this structure predispose patients to disc herniation, wherein fragments of disc tissue herniate through this annular defect causing neural compression and radicular pain(19).

As the mechanical and structural integrity of the disc progressively deteriorates neurovascular invasion may occur contributing to the development of back pain(20). In the following sections the aetiological factors and pathophysiology of intervertebral disc degeneration will be briefly explored.

1.4.1. Mechanical Effects

With respect to mechanically loading the intervertebral disc there is a delicate balance between "normal" mechanical loading, which is required for maintenance of an optimal disc cellular phenotype(21,22), and excessive mechanical loading that causes damage. Excessive loading can result from excessive bodyweight(23) or trauma and produces many of the features of degeneration that can be visualized by histological and radiological methods.

1.4.2. Genetic Effects

Familial predisposition to disc degeneration has been observed in numerous studies (24-28). As age progresses genetic effects are more evident and are affected by environmental influences, such as trauma and smoking(29). Polymorphisms of genes including aggrecan, collagen, and matrix degrading enzymes have been implicated in the degenerative process(30-32).

1.4.3. Cellular Changes

Studies comparing degenerate discs with non-degenerate controls have demonstrated increased evidence of senescent cells in degenerate intervertebral discs(33). Such cells lose the ability to divide, thus potentially contributing to the decreased cellularity of the diseased degenerate intervertebral discs. Moreover, the senescent cells have a reduced ability to function. Thus, they produce less matrix

which, in turn further compromises the structure of the intervertebral discs.

1.4.4. Nutritional Effects

Intervertebral discs comprise the largest essentially avascular tissue in the human body. Only the outermost layers of the AF contain blood vessels. The cells of NP are dependent on diffusion of nutrients from capillary buds in the cartilaginous endplate to meet their metabolic needs(18). The cells in the NP are therefore metabolically compromised by this limited vascular and nutritional supply and may promulgate intervertebral disc degeneration. Causes of impaired nutrition to the intervertebral disc include endplate calcification, microvascular disease and smoking and have all been associated with early disc degeneration.

1.4.5. Matrix Changes

Ultimately there is an imbalance between the rates of production and breakdown of the matrix components leading to a cascade of events (see Figure 2) consisting of alterations in matrix synthesis, reduced aggrecan synthesis and a transition of collagen production from type II to type I(34). Further, a reduction in cellular viability and activity together with alterations in the cytokine profile reduce proteoglycan content. This cascade leads to dehydration and loss of mechanical integrity of the intervertebral disc(17,18).
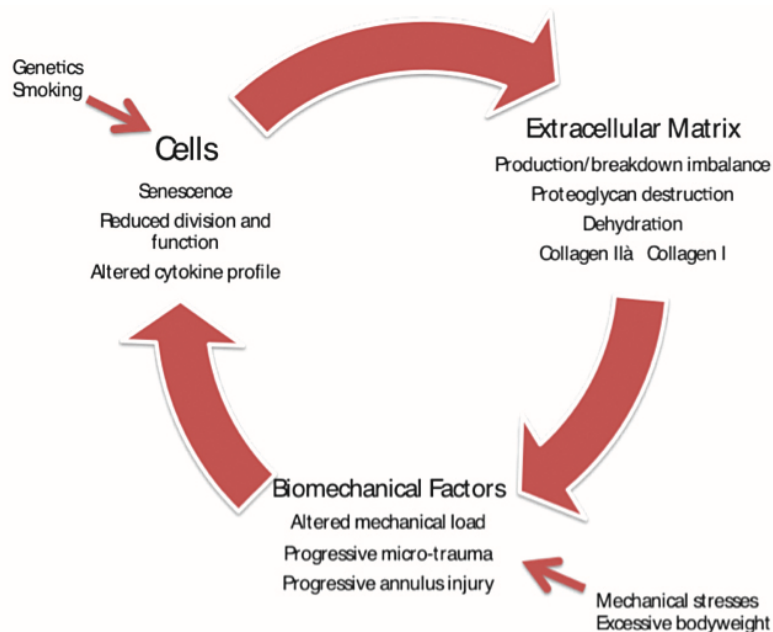

Figure 2. Schematic of the process of disc degeneration demonstrating multifactorial pathophysiology and interplay of cellular, matrix and biomechanical factors. Figure from Daly et al.(35) and a modification of figure from Vergroesen et al.(36).

1.4.6. Mechanical Changes

The dehydration of the intervertebral disc reduces the mechanical support provided by the swelling pressure of the previously hydrated NP. This alters the mechanical load to which the AF is exposed and thus the tension in the AF collagen fibres. This leads to subsequent progressive microtrauma of these fibres. (17). The degeneration of the AF and subsequent tears in this structure predispose patients to disc herniation, wherein fragments of disc tissue herniate through this annular defect causing neural compression and radicular pain(19).

1.4.7. Neural and Vascular Changes

As the mechanical and structural integrity of the disc progressively deteriorates neurovascular invasion may occur via annular tears. Neurovascular invasion extending to the NP via annular fissures has been demonstrated in painful discs in clinical studies(37). In contrast, control (non-painful) discs demonstrated restriction of vascular and neural supply to the outer annulus(37). This process of neoinnervation of the degenerate intervertebral discs is hypothesized to be a significant contributor to the development of back pain(20)

1.4.8. Conclusion

Intervertebral disc degeneration is a complex, multifactorial pathophysiological process. Given the importance of disc degeneration in the development of back pain the importance of appropriate preclinical models cannot be overstated. In addition to effectively modelling the degenerative process described above an appropriate animal model of the post lumbar discectomy intervertebral disc should also recapitulate the annular and nuclear defect that defines this setting. The choice of an appropriate animal model for the post lumbar discectomy intervertebral disc will be explored below.

1.5. Animal Models of Intervertebral Disc Degeneration

The development of appropriate animal models of intervertebral disc disease is imperative to gain insight into its pathophysiology and to develop and test potential therapies. In vitro and in silico (computer based) systems can be helpful to investigate specific components of intervertebral disc degeneration. However, given the complexity inherent to the intervertebral disc with biochemical, biomechanical, nutritional and metabolic factors acting simultaneously, in vivo animals are able to more faithfully replicate this environment. A range of animal models and mechanisms of replicating the process of degeneration have been investigated and utilized in efforts to develop appropriate models of intervertebral disc degeneration. However, given the extreme complexity of this system no perfect model currently exists.

1.5.1. Existing Animal Models

Animal models of intervertebral disc degeneration range from small rodents such as mice knockout models[38], to rats[39], rabbits[40], dogs[41], goats[42], sheep[11] and primates[43]. Various mechanisms of inducing degeneration have been described for these animal models which are summarized in Table 1. Although providing a variety of mechanisms of inducing degeneration relatively few of these models provides an appropriate model of the post lumbar discectomy intervertebral disc. The characteristics of an ideal animal model of the post lumbar discectomy intervertebral disc will be explored below.

Table 1 Summary of animal models of disc degeneration. Taken from Daly et al.(35)

Animal	Notochordal cells in adult intervertebral disc	Mechanism	References
Mouse	Present	*Spontaneous*	
		Knockout- Col2a1 gene/Type II Collagen	Sahlman et al.(38)
		Collagen IX mutation	Kimura et al.(44)
		Mechanical	
		Tail bending	Court et al.(45)
		Bipedal mouse	Higuchi et al.(46),
		Instability- resection	Goff et al.(47)
		of posterior elements	Miyamoto et al.(48)
Rat	Present	*Spontaneous*	
		HLA-B27 and human β_2-microglobulin gene transgenic	Hammer et al.(49)
		Mechanical	
		Tail bending	Lindblom(50)
		Bipedal rat	Goff et al.(47)
		Ilizarov-type apparatus	Iatridis et al.(51)
		Cyclical compression	Ching et al.(52)
		Structural	
		Stab incision	Rousseau et al.(53), Jeong et al.(54)

Sand Rat	Present	*Spontaneous*	
		Laboratory diet	Silberberg et al.(55), Gruber et al.(56), Moskowitz et al.(57)
Rabbit	Present	*Mechanical*	
		External loading device	Kroeber et al.(58)
		Adjacent segment fusion	Phillips et al.(59)
		Structural	
		Annulus puncture	Masuda et al.(40)
		Chemonucleolysis-chondroitinase ABC	Kiester et al.(60)
		NP aspiration	Sakai et al.(61)
Canine			
Non-Chondrodystrophoid Dog	Present	*Spontaneous*	Bergknut et al.(41)
		Structural	
		Annular injury with scalpel/drill	Keyes and Compere(62)
		Subtotal discectomy	Hohaus et al.(63)
Chondrodystrophoid Dog	Absent	*Spontaneous*	Gillett et al.(64), Bergknut et al.(41)
		Structural	
		Needle aspiration of NP	Serigano et al.(65)
		Chemonucleolysis-Chymopapain	Melrose et al.(66)

Goat	Absent	*Structural*	
		Chondroitinase ABC	Hoogendoorn et al.(67)
		Drill-bit injury/annulotomy	Zhang et al.(42)
Pig	Present	*Structural*	
		Nucleotomy	Acosta et al.(68)
Sheep	Absent	*Structural*	
		Partial thickness annulotomy	Osti et al.(69), Oehme et al.(70), Melrose et al.(71)
		Annular incision and partial nucleotomy (simulated microdiscectomy)	Oehme et al.(11)
		Chondroitinase ABC	Ghosh et al.(72)
Macaque	Present	*Spontaneous*	
		Age related degeneration	Nuckley et al.(73)
Baboon	Present	*Spontaneous*	
		Age related degeneration	Lauerman et al.(74), Platenberg et al.(43)
Rhesus Monkey	Present	*Structural*	
		Annulotomy +/- collagenase	Stern et al.(75)
		Bleomycin injection of subchondral bone	Wei et al.(76)

NP indicates nucleus pulposus

1.5.2. Properties of the Ideal Animal Model

1.5.2.1. Notochordal Cells

The vertebral column and thus intervertebral discs of all mammals arise from aggregation of the mesenchyme around the notochord and subsequent segmentation during development(77). Notochordal cells persist in the NP of the majority of species (e.g. mice, rats, rabbits and pigs) into adulthood. However, the number of these cells decreases rapidly following birth in humans and notochordal cells are completely absent from the NP by early adulthood(77). Sheep and goats are among the few animals to also lose the notochordal cells rapidly from the NP following birth. Dogs are divided into two populations with regard to notochordal persistence into adulthood. Chondrodystrophoid (CD) dogs rapidly lose the notochordal cells following birth and as such are predisposed to intervertebral disc degeneration in later life. Non-chondrodystrophoid (NCD) dogs have persistent notochordal cells and are far less inclined to disc degeneration. The persistence of notochordal cells is an important consideration as these cells have a significant influence on the intervertebral disc by influencing proteoglycan metabolism (78,79), hyaluronan production(80), and possible progenitor cell function(77).

Animal models with persistent notochordal cells into adulthood may be observed to lose these cells following adequate stimulus(81,82). Apoptotic processes have been demonstrated to play a significant role in this process of notochordal cells loss(82,83) and are also observed in human aged and degenerate discs(84).

However, given the use of animal models to investigate cellular regenerative therapies for the treatment of disc degeneration the potential presence of a pre-existing precursor cell population may complicate investigation of the regenerative potential of such therapies. For instance, in cell transplantation therapies, one cannot be sure that it is not the resident notochordal cells which are responsible for the regenerative effects, instead of, or in combination with, the transplanted cells. Thus, an optimal animal model of intervertebral disc degeneration should demonstrate the absence of notochordal cells in the adult intervertebral disc.

1.5.2.2. Disc Size and Geometry

Intervertebral disc size and geometry vary markedly across species and according to location within the spine. The discs of most animal models are smaller than human intervertebral discs. Disc size affects solute diffusion in the intervertebral disc. Given the largely avascular nature of the intervertebral disc and dependence on diffusion to meet nutritional requirements this is of particular significance to the clinical relevance of animal models. Given the size discrepancies between common animal models and

humans, investigators have analyzed disc geometry hoping to better determine the relevance of particular models to the human intervertebral disc(85)

1.5.2.3.   Disc Mechanical Forces

The vast majority of animal models of intervertebral disc disease are quadrupedal. The only bipedal models available are certain primates (e.g. rhesus monkey(86)) and the bipedal mouse and rat models(47). The use of primates, present ethical dilemmas that preclude their usage in most institutions. The bipedal mouse and rat models also present ethical issues given the requirement for forelimb amputation. Furthermore, questions have been raised regarding the validity of this model as bipedal rats have been observed to spend no more time in an upright position than quadrupedal rats(87).

The discrepancy between quadrupedal animal models and the bipedal human may be less significant than initial impressions suggest. As studies have shown that a significant contributor to IVD loading is muscle contraction and ligament tension(88,89), which may be greater in large quadrupedal animals such as sheep than humans due to the challenge of stabilizing a horizontally aligned spine(77), overall IVD loading may actually be increased in such animal models relative to the clinical context(90). This is reflected in the increased bone mineral density observed in large quadrupedal animals relative to humans(91). These and other observations from biomechanical studies have lead authors to promote the validity of the ovine model as a preclinical model of the human intervertebral disc(92).

1.5.2.4.   Sheep Models

After considering the above properties of the ideal animal model, the sheep or ovine model has proven to have particular merit for several major reasons. Firstly, the sheep, similar to humans, suffers from a loss of notochordal cells in early adulthood, predisposing the sheep intervertebral discs to degeneration(93). The age at which ovine intervertebral disc notochordal cells are lost is not known, but they are already lost by the age of four years(93). The sheep is of a roughly similar size to humans and, despite its quadrupedal stature, demonstrates many biomechanical similarities to the human intervertebral disc(92). The sheep is a hardy animal with demonstrated ability to tolerate surgical intervention. The ovine spine has therefore been used extensively to model disc degeneration(69-71) , test implant devices and in the preclinical investigation of cellular therapies(11,94-96).

1.5.3.   Mechanisms of Injury

Given the complexity of human disc degeneration no animal model can perfectly mimic the entire pathophysiological process. Disc degeneration in animal models can be initiated by chemical insult (e.g. chondroitinase-ABC injection(72)) or mechanical stimuli (e.g. surgical incision(70), nucleotomy-NP aspiration(97), drill bit injury(42)) though can develop spontaneously in some animals(98). Each of

these mechanisms have particular merits with regard to the investigation of intervertebral disc degeneration. For the purpose of creating a model of the post lumbar discectomy intervertebral disc the structural model has particular appeal as the post-microdiscectomy lumbar disc has suffered spontaneous and surgical compromise of the annulus fibrosus and nucleus pulposus. Surgical injury to the intervertebral disc is a well-established method of inducing disc degeneration.

Annular injury models were first described in the 1930s by Keyes and Compere(62). Keyes and Compere demonstrated that annular injury with a scalpel with subsequent NP expulsion lead to loss of disc height and degenerative changes at the index level. Following these pioneering studies multiple intervertebral disc injury methods have been investigated for their potential to induce disc degeneration. Broadly such methods can be considered under the categories of partial thickness annular injury and full thickness annular injury with nucleus involvement (see Figure 3). Full thickness annular injuries have the advantage of producing nuclear avulsion with relatively rapid degeneration. Partial thickness injuries produce a slower degenerative process.
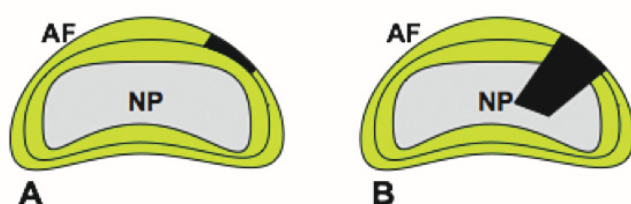

Figure 3. (A) Partial thickness annular injury. (B) Full thickness annular injury with NP involvement. AF indicates annulus fibrosus; NP indicates nucleus pulposus. From Daly et al.(35)

Stab injuries and annulotomies have been performed in a variety of animal models including rats(53), rabbits(40), sheep(69) and pigs(68). Osti et al.(69) demonstrated in an ovine model that partial thickness annular injury, consisting of a 5mm depth incision that left the inner annulus and NP intact at the time of injury, would lead to progressive failure of the inner annulus with progressive disc degeneration over several months. Oehme et al.(70) demonstrated in an ovine model that after three months a larger (20mm x 6mm) partial thickness annular injury resulted in significantly increased disc height loss, increased MRI Pfirrmann degeneration grades, increased histological injury scores and decreased NP glycosaminoglycans in the injured discs.

Full thickness intervertebral disc injury is demonstrated in the approach of Oehme et al.(11). In this injury model a simulated partial lumbar microdiscectomy was performed by creating a 3x5 mm annular incision in ovine discs followed by removal of 200 mg of intervertebral disc tissue, including NP. 24 weeks following performance of the partial-microdiscectomy injured and otherwise untreated

intervertebral discs demonstrated increased disc height loss, increased MRI Pfirrmann degeneration scores and reduced NP proteoglycan content relative to controls.

A novel full thickness intervertebral disc injury caprine model utilizing a drill bit has recently been described by Zhang et al.(42). The authors compared scalpel blade annulotomy with insertion of a 4.5mm drill bit to a depth of 15mm. At two months the drill bit injured intervertebral discs demonstrated significantly increased histological injury scores relative to controls. Radiologically there was no significant difference observed between the drill injured, stab incision injured and control discs on 1.5T MRI. Biochemical analysis was not performed. Thus, on histological analysis the large animal models described by Zhang et al.(42) represented a highly replicable mode of full thickness intervertebral disc injury.

The full thickness annular injury methods described by Oehme et al.(11) and Zhang et al.(42) both represent methods of inducing intervertebral disc degeneration with creation of annular and nuclear defects that better mimic the changes in intervertebral disc degeneration. Both models have demonstrated the ability to promote intervertebral disc degeneration in large animals with similarities to the human intervertebral disc. As such these two methods of inducing intervertebral disc degeneration warrant particular consideration for their potential to replicate the post lumbar discectomy intervertebral disc.

Having explored the pathophysiology of intervertebral disc degeneration and characterized the ideal animal model of the post lumbar discectomy intervertebral disc it is important to explore the clinical sequelae of intervertebral disc degeneration and the status and failings of existing therapies.

1.6.   Clinical Outcomes of Lumbar Intervertebral Disc Degeneration

Clinically relevant outcomes of lumbar disc degeneration include back pain and symptoms and signs attributable to neural compression, such as radiculopathy. Chronic low back pain is the leading cause of disability worldwide, is associated with significant reduction in quality of life and may be associated with psychiatric illness, such as depression and anxiety(99). Chronic low back pain is unfortunately a relatively common outcome following lumbar microdiscectomy, occurring in up to one third of patients(5) and of sufficient severity to be disabling in close to 10%(6).

1.6.1.   Back Pain

Lower back pain is the most common clinical outcome of disc degeneration. Pain derived from the disc itself is termed discogenic pain(100-103). However, the pain generator may also arise from adjacent structures such as the facet joints, periosteum, ligaments and paraspinal musculature(104). Clinically, it can be difficult to identify the cause of back pain. Back pain will be experienced by 75-80% of people

at some stage in life with prevalence ranging from 15-45%(105). Severe radiological degeneration is associated with a two-fold increase in chronic lower back pain(101,106). However many asymptomatic patients will have radiological evidence of degeneration(103,107). Radiologically differentiating the degenerate spine from the ageing spine is difficult. Prevalence of back pain increases with age from the third decade until the 60-65 year age group and then gradually declines(108).

Discogenic back pain remains controversial. Many patients with radiological disc degeneration do not suffer back pain and, conversely, many patients with back pain do not have radiological disc degeneration. However, large population based studies have demonstrated a significant correlation between back pain and the presence of disc degeneration on MRI(101). Extent of radiological degeneration correlates with severity of pain and disability, particularly in younger patients(109). Disc height changes have been demonstrated to correlate with low back pain(110). Degenerative endplate changes, termed Modic changes on MRI, are also strongly associated with back pain(111).

The discogenic theory of back pain garners additional mechanistic and histological support from clinical studies demonstrating the pathological ingrowth of blood vessels and nerves fibres in degenerate discs- thus providing an anatomical pathway for the mediation of pain(112-114). In addition, pro-inflammatory mediators capable of augmenting or contributing to the pain process have also been identified in herniated lumbar discs(115). Interleukin-8 and Tumor Necrosis Factor-alpha were identified as potentially playing a significant role in the development of discogenic pain(116).

A gold-standard investigation for determining discogenic pain has yet to be determined. Provocative discography was previously popular, however, discography has been demonstrated to accelerate disc degeneration, disc herniation, loss of disc height and the development of reactive endplate changes(117). As such it is not practiced routinely in many spine centres.

1.6.2. Symptoms Related to Neural Compression

Neural compression may lead to radiculopathy, a dysfunction of a nerve root causing neurogenic pain (sciatica), weakness, reflex and/or sensory abnormalities in the distribution of the nerve. Radiculopathy is a common clinical presentation of lumbar intervertebral disc herniation producing neural compression. Cauda equina syndrome is a condition in which compression of the cauda equina, most commonly as the result of a large herniated disc, leads to neurological deficits. Sciatica has a lifetime incidence of 13-40%, however up to 86% of episodes will resolve with conservative management (106,118).

1.6.3. Treatments For Degenerative Disc Disease and Their Outcomes

1.6.3.1. Non-Operative Treatments

For many patients conservative management is the first line of therapy for symptomatic lumbar intervertebral disc degeneration. The general consensus remains that in the absence of a neurological deficit or cauda equina syndrome a trial of conservative management should precede surgical intervention (119,120). Conservative management, however, is not clearly defined and may vary from simple analgesia alone to physiotherapy and non-surgical intervention such as nerve root injection. The most cited study regarding pure conservative management for degenerative disc disease with radicular pain is that of Saal and Saal in 1989(121) in which 90% of patients demonstrated good or excellent outcomes with conservative management. However, of the 347 consecutive patients identified only 64 were followed for 31.1 months. As such the study has been justly criticized for its poor retention of enrollees.

1.6.3.2. Surgical Treatments

Lumbar Microdiscectomy

Lumbar discectomy is performed for patients with symptomatic lumbar disc prolapse refractory to conservative management. The procedure consists of a small laminotomy with removal of the herniated intervertebral disc performed to decompress the symptomatic nerve. The objective of the procedure is to treat radicular symptoms and is not directed at resolving back pain, although such improvements have been reported in some studies.

More than 80% of patients will report improvement of radicular symptoms following lumbar discectomy. Unfortunately, lumbar discectomy, while relieving the radicular symptoms, fails to address the underlying pathophysiology of disc degeneration that contributed to the annular failure. Consequently, up to one third of patients continue to experience back pain(5) that may be disabling in up to 10%(6). Recurrent disc herniation occurs in up to 15% of patients(7). A recent retrospective analysis of US national insurance databases revealed that 12% of patients subjected to microdiscectomy will undergo reoperation for the same procedure within four years(8). Approximately 40% of these patients will subsequently progress to fusion surgery within four years(8).

It is this failure of lumbar discectomy to halt disc degeneration and the subsequent adverse sequelae that highlights the need for the development of regenerative therapies. In addition to developing a regenerative therapy that can be administered at the time of lumbar discectomy, opportunity exists to improve the lumbar discectomy procedure by optimization of post-operative care following this operation.

Lumbar Discectomy Post-Operative Care

Lumbar microdiscectomy in contemporary practice is minimally invasive, patients typically mobilize the same day and are discharged home the following day, making the operation suitable for day-procedure(122). Traditionally following surgery, patients have been advised to restrict sitting, lifting or resuming other activities of everyday life, and are advised to either stand or lie for variable periods (123). Sitting imposes greater intra-discal pressure than does standing(124) though evidence that increased pressure increases disc reherniation risk is lacking.

Such restrictions impact upon patients' ability to return to work, travel or drive and basic comfort. It has been suggested that activity restrictions may also raise patient anxiety regarding reherniation risk. Moreover, surgical practice regarding activity restriction varies, the dearth of evidence resulting in absence of clear clinical guidelines for surgeons, nurses, physiotherapists and occupational physicians.

Two prospective studies published in the 1990s reported incidence of symptomatic recurrent disc protrusions and reoperation, and time to return to work in a cohort of patients whose movement was not restricted post lumbar microdiscectomy. Compared to rates in the literature among movement-restricted patients, adverse outcomes in this cohort were not considered higher(125,126). However, without randomisation and with the absence of a control group, the evidence from such studies is relatively weak.

Bono et. al.(127) published the first report of a randomised controlled trial investigating post-operative activity restrictions following lumbar discectomy. This trial compared post-operative protocols consisting of short (two weeks) and long (six weeks) periods of activity restriction following lumbar discectomy. The authors observed no significant difference in outcome as assessed by Visual Analog Scale (VAS) back or leg pain or Oswestry Disability Index (ODI). Disc reherniation rates differed between the groups observing short (11%) and long (7%) periods of activity restriction. Though, this difference did not achieve statistical significance and did not translate into an appreciable difference in clinical outcome. However, the authors conceded that their study was underpowered to detect a significant difference in disc reherniation rate and calculated approximately 800 patients per arm would be required to achieve sufficient statistical power.

All previous studies on post-operative restrictions following lumbar discectomy have relied on self-reported adherence to mobility restrictions. Non-adherence is a well-recognized phenomena in spine surgery trials, for example the non-adherence rate in the SPORT trial was approximately 40% at one year(128). Such outcomes are therefore likely to be biased. Contemporary wearable electronic devices that can accurately record the patient's position (i.e. sitting/standing) enable empirical observation of

patient adherence to a regimen of sitting restrictions with great reliability.

The question of the role, if any, for post-operative activity restrictions following lumbar discectomy remains unanswered. The studies of Carragee et al.(125,126) and Bono et al.(127) highlight the importance of this question. With the advent of contemporary wearable devices that monitor patient activity such questions may now be addressed accurately without solely relying on the vagaries of self-report.

Lumbar Fusion

The rationale underlying spinal fusion is to eliminate the painful segmental motion(129). Spinal fusion consists of stabilising a segment of the spine to allow bony union to occur thus immobilising that section of spine. Degenerative indications for lumbar fusion include instability, such as due to spondolylisthesis or when a wide neural decompression is required causing iatrogenic instability. Back pain due to lumbar degenerative disc disease is a contentious indication for spinal fusion. This contention has led to the National Institute for Health and Care Excellence (NICE) of the United Kingdom publishing guidelines that fusion for the indication of non-specific low back pain should be performed only as part of a randomised controlled trial and lumbar disc replacement should not be performed at all(10,130).

The reasons for this contention regarding fusion surgery for back pain include the difficulty identifying the symptomatic disc or pain generators, persistence of pain and disability in many patients following fusion surgery and conflicting evidence regarding the efficacy of fusion surgery(131,132). Several randomised controlled trials demonstrate improvements in pain and disability following fusion surgery for back pain(133). However, other studies have demonstrated no significant difference between surgery and an interdisciplinary program of physical and psychological rehabilitation(134-136). A 2013 meta-analysis concluded support for fusion for back pain where a diagnosis of disc degeneration can be made(133). In this pooled analysis of 3060 patients 71% of patients were satisfied with their outcome. This perspective was confirmed in a 2017 meta-analysis and systematic review which reported that fusion performed for low back pain resulted in greater pain relief and satisfaction than non-operative management(137).

A variety of surgical techniques are available to promote fusion surgery: posterior lumbar interbody fusion (PLIF), anterior lumbar interbody fusion (ALIF), direct lateral interbody fusion (DLIF), etc. (138). However, central to the majority of contemporary lumbar fusion operations is implantation of a prosthesis into the intervertebral disc space combined with a method of immobilization, e.g. pedicle or vertebral body screws connected to rods. This combination fixes the segment to maximize the probability of bony fusion occurring.

Unfortunately, fusion surgery is not the panacea for discogenic back pain. A failure of fusion, pseudoarthrosis, occurs following approximately 11% of lumbar fusion procedures(133). This non-union is a key contributor to persisting chronic back pain, disability and failed back syndrome and a significant contributor to 12.5% of patients undergoing reoperation(133). Furthermore, even if fusion is successful, the adjacent levels can degenerate which leads to further pain and the possible need for further surgical intervention. This is because fusion alters the biomechanical forces to which the adjacent intervertebral segment is exposed and, increases the likelihood of degeneration of the adjacent disc/s(59,139) . Radiographic Adjacent Segment Degeneration (ASD) has been detected in 26.6% of patients post fusion, whilst symptomatic adjacent segment disease occurs in 12.1% of patients at five years(139). The development of adjacent segment disease and the perceived failings of fusion surgery as detailed above have led to the development of a surgical alternative intended to preserve motion at the index level, namely lumbar total disc arthroplasty.

Lumbar Total Disc Arthroplasty

Lumbar total disc arthroplasty (TDA) involves insertion of a motion-preserving, non-biological, artificial prosthesis into the disc space. The indication for TDA is axial lower back pain with stable single level intervertebral disc disease(140). The degenerate disc is removed entirely and replaced with the artificial prosthesis which anchors to the adjacent vertebral bodies producing an artificial articulation.

The long-term benefit of lumbar TDA remains unclear(141). Series with follow-up extending ten or more years have given mixed results with success rates of up to 82% at 10 years cited by one investigator(142) while others report reoperation rates approaching 40% at 10 years for single level lumbar disc replacement (143). Furthermore, the study with the longest follow-up to date, an average of 17 years, demonstrated that 60% of patients with TDA underwent spontaneous fusion or ankylosis(144) and that these patients had better outcomes than those with functional implants. This study was retrospective and has been criticized due to suboptimal device placement, undersized prototype devices and incorrect statistical calculations(145-147), however, it did highlight the lack of knowledge regarding the long-term outcome of this prosthesis. Over the short term (one to five years) lumbar TDA has been demonstrated to reduce back pain with equivalency to lumbar fusion techniques in most studies(145,148,149). One recent meta-analysis of randomised controlled trials with two years of follow up suggested superiority of TDA over fusion with respect to disability, pain, patient satisfaction, overall success, complications and narcotic usage outcomes(9).

The efficacy of TDA in preserving motion and reducing adjacent segment disease over the long term is unknown. Motion preservation at the index level has been demonstrated in up to 92% of patient at five years(149). A reduction in radiological adjacent segment degeneration at five years in TDA vs fusion

has been reported(150), however, a reduction in symptomatic adjacent segment disease has yet to be demonstrated(151). TDA has demonstrated superiority in maintaining adjacent level disc pressure and facet forces in biomechanical studies(152).

Despite evidence of improvement in radiological outcomes and advantages in biomechanical studies TDA remains a non-biological, destructive and ultimately flawed attempted solution to discogenic pain. Lumbar fusion and total disc arthroplasty, when performed for the treatment of discogenic low back pain, represent imperfect attempts to address a complex pathophysiological entity via relatively crude mechanical means. As detailed previously, a proportion of patients undergoing lumbar fusion, do so as a result of the progression of disc degeneration following lumbar discectomy(8). This patient group, in particular, would benefit from regenerative therapy delivered at the time of the index lumbar discectomy procedure to alter the degenerative process.

1.7. Cellular Therapy

Given the invasiveness of existing surgical therapies and the inability to treat the underlying degenerative disc disease, research groups throughout the world have investigated experimental treatments. Cellular therapy has been investigated for the treatment of symptomatic intervertebral disc degeneration. A variety of cells have been investigated as potential sources of cellular therapy however the majority of investigators have focused their efforts on stem cells and chondrocytes.

1.7.1. Intervertebral Disc Chondrocytes

Intervertebral disc nucleus pulposus "chondrocyte like cells" were one of the first cell sources investigated for the potential for intervertebral disc regeneration (153-155). The reduction in the number and activity of disc chondrocytes in matrix production and maintenance is a significant contributing factor to disc degeneration(156).

Preclinical studies in various animal models have demonstrated the ability of both cultured autologous and allogeneic nucleus pulposus chondrocytes (NPCs) to promote disc regeneration as assessed by measures such as disc height index, MRI appearance, histological analysis and biochemical assay(63,157,158). One of the earliest preclinical investigations of the potential of cell therapy to promote intervertebral disc regeneration involved the administration of autologous disc chondrocytes in a canine model of disc injury(154,155). This study demonstrated the capacity of autologous culture expanded nucleus pulposus chondrocytes to promote intervertebral disc regeneration following percutaneous administration as assessed by disc height index, gross morphological and histological analysis. In a subsequent study by Hohaus et. al.(63) canines that underwent annular injury and partial nucleotomy followed by autologous nucleus pulposus chondrocyte injection demonstrated viable disc cells, production of extracellular matrix similar to normal intervertebral disc tissue and statistically

significant retention of disc height. These successful preclinical trials lead to the EuroDISC clinical trial of Autologous Disc Chondrocyte Transplantation (ADCT)(159) in which patients planned for single level lumbar microdiscectomy underwent collection of nucleus pulposus tissue from the affected disc at the time of operation. The autologous NPCs subsequently underwent tissue culture expansion and percutaneous implantation into the index disc 12 weeks later. Interim analysis of 28 patients at 24 months revealed the patients who received autologous expanded NPCs had significantly reduced back pain, reduced disc height loss and increased adjacent disc hydration on MRI compared to control(159). ADCT has been available in Germany since 1997 but has yet to achieve FDA approval(140,154). The ADCT approach has evolved further via addition of the autologous cultured cells to an injectable, in-situ polymerizing, modified albumin, hyaluronic acid hydrogel (NOVOCART). During Phase I investigations NOVOCART was investigated in the treatment of post lumbar discectomy intervertebral discs and adjacent degenerate discs(160,161). Ninety days following lumbar discectomy 12 patients underwent ADCT in NOVOCART via percutaneous administration. In this cohort one patient underwent reoperation for recurrent herniation within seven months. The efficacy of this therapy as assessed by standardized pain and disability instruments are yet to be reported.

A modification of the transplantation of autologous nucleus pulposus cells was investigated by Mochida et al.(162). Nine patients with a moderately degenerate (Pfirrmann Grade III) disc adjacent to a lumbar intervertebral disc scheduled for posterior lumbar interbody fusion received autologous nucleus pulposus cells "activated" by co-culture with autologous bone marrow derived mesenchymal stroma cells. These cells were administered seven days after NP cell harvest and fusion surgery. In vitro studies demonstrated co-culture resulted in increased cell-proliferation. On clinical follow-up to five years there was no radiological deterioration of the cell treated levels and all patients reported no back pain.

The approaches detailed above demonstrate the potential feasibility of cellular therapy to promote intervertebral disc regeneration while also highlighting the limitations inherent in the autologous chondrocyte approach. Autologous disc chondrocytes must be harvested at the time of microdiscectomy and subsequently expanded. This limits this procedure to only patients already undergoing intervertebral disc surgery; it also requires an additional intervention at another time. The disc cells are taken from the degenerate level and thus may have reduced function or already be affected by the degenerative process(33). The modification of Mochida et al. involving co-culture with autologous mesenchymal stroma cells(162) is a novel approach to addressing this particular limitation but also serves to highlight the sub-optimal nature of chondrocytes from degenerate discs. Furthermore, this autologous approach would require Good Manufacturing Practice (GMP) facilities to be available to the hospital, which may be prohibitively expensive for many institutions. Ultimately, the

use of autologous disc chondrocytes as a cell source for intervertebral disc degeneration presents many challenges likely to preclude widespread adoption of this approach.

Investigators have explored alternative methods and sources for acquiring chondrocytes in order to obviate many of these challenges. Non-disc derived autologous chondrocytes, from auricular cartilage, have been investigated pre-clinically and demonstrated to survive in a rabbit model of disc regeneration and furthermore lead to production of hyaline cartilage in the nucleus pulposus(163). This approach, however, has yet to progress to clinical trials and would also entail two separate interventions. Another approach utilized percutaneous administration of banked allogeneic juvenile chondrocytes from cadaveric donors in 15 patients with low back pain and single level lumbar disc degeneration on MRI (Pfirrmann Grade III-IV) (164). Over 12 months follow-up mean ODI and SF-36 scores all improved from baseline. Additionally, at six months follow-up 10 patients demonstrated improvements in MRI. Administration of juvenile chondrocytes avoids the requirement for multiple interventions. However, acquiring a sufficient supply of juvenile chondrocytes on an ongoing basis may prove challenging.

1.7.2. Notochordal Cells

Notochordal cells have been investigated for their potential to contribute in the treatment of intervertebral disc degeneration. However, as these cells are absent from within the human adult intervertebral disc and thus must be obtained from immature donors, practical considerations are likely to preclude their adoption clinically. Investigators have recently demonstrated the ability of notochordal cell conditioned medium to protect human nucleus pulposus cells from stress induced apoptosis(165). Such approaches may provide an alternative route of benefiting from the biological activity of the notochordal cells, however, do not solve potential sourcing challenges.

1.7.3. Stem Cells

Given the challenges associated with the use of autologous chondrocytes, as a cell source for regenerative therapies for disc degeneration, a variety of stem cell based therapies are under investigation. A stem cell is distinguished by its ability to self-renew and differentiate into a variety of tissue or organ specific cells under the appropriate conditions. This definition encompasses a broad and heterogeneous variety of cells derived from a variety of tissues, by different methods. Broadly, stem cells can be classified as embryonic stem cells or adult stem cells, although induced pluripotent stem cells must also be considered as an additional category.

1.7.4. Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from the human embryo blastocyst inner cell mass and are regarded as totipotent, meaning they can differentiate into cells of all tissues(166,167). This totipotency is unfortunately associated with tumorigenicity, as embryonic stem cells have the capacity to produce

teratomas(168). Additionally, the use of embryonic stem cells is associated with significant legal and ethical considerations which are likely to preclude adoption in the treatment of degenerative spine conditions. As such despite demonstrating the ability to promote notochordal cell formation in a rabbit model of intervertebral disc injury(169), ESCs are unlikely to play a significant role in the treatment of degenerative disc disease in the near future.

1.7.5. Other Types of Stem Cells

Other stem cells, namely human amnion epithelial cells (hAECs) derived from the amniotic tissue, and stem cells obtained from human umbilical cord blood or tissues, have also been used in pre-clinical studies to treat intervertebral disc degenerative conditions(95,170). The ready availability of these cells from discarded pregnancy tissue, and the development of large cell banks to store such cells may make them a convenient source of stem cells for the use in many applications. This includes degenerative spinal applications, should animal studies demonstrate efficacy. Our group have evaluated the ability of hAECs to enhance cervical interbody fusion, however, they were found to be inferior to MPCs in promoting fusion(95). To our knowledge AECs have not yet been used in intervertebral disc cartilage regenerative strategies or clinical spine studies. In distinction to amniotic cells, cryopreserved amniotic membrane (cAM) has been applied to the annular defect following lumbar microdiscectomy in a randomised controlled trial(171). Patients who received cAM demonstrated significantly better ODI and SF-12 scores at 6 weeks and 24 months compared to controls, but not at the other timepoints: two weeks, six months and one year. Interestingly, the cAM group experienced no disc reherniation (0/40) while the control group experienced three intervertebral disc reherniations at the index level. The authors hypothesized that the efficacy observed for cAM in improving ODI, SF-12 and reherniation rates may be secondary to the anti-inflammatory, regenerative and anti-scarring activities ascribed to a unique glycoprotein complete called the HC-HA/PTX3 complex(172). Umbilical MSCs have also been investigated as a potential source of cell for intervertebral disc regeneration but will be addressed with other MSCs.

1.7.6. Induced Pluripotent Stem Cells

Induced pluripotent stem cells are somatic cells reprogrammed to produce an embryonic stem-cell like state. iPSCS were first reported in 2006 in a mouse model(173). iPSCS have subsequently been investigated as a potential cell source for intervertebral disc regeneration. The ability of mouse induced pluripotent stem cells to differentiate into cells with a nucleus pulposus like phenotype was demonstrated by Chen et al. (174). However, given concerns regarding the potential tumorigenicity secondary to pluripotency(175) it seems less likely that iPSCs will play a role in the treatment of degenerative spine conditions.

1.7.7. Adult Stem Cells

Adult stem cells are progenitor cells present in most tissues of the body, with a putative role in tissue repair and restoration. Adult stem cells have been discovered and characterized in a broad range of tissues: bone marrow, adipose, periosteum, synovial membrane, muscle, skin and blood(176-179). Bone marrow derived stem cells can be divided into haematopoietic stem cells (HSCs) and mesenchymal stem cells. The majority of research investigating potential sources of adult stem cells for the treatment of degenerative spine conditions has focused on mesenchymal stem cells although other types of stem cells have been studied.

1.7.8. Haematopoeitic Stem Cells

Haematopoietic Stem Cells are bone marrow derived stem cells that produce the cells of the haematopoietic lineage. These cells have been used for decades in the clinic to treat haematological malignancies. A small clinical study investigating the administration of haematopoietic cells for lumbar intervertebral disc degeneration was reported in 10 patients(180). No patient reported an improvement in back pain and 80% of patients underwent surgery within one year of the HSC administration. There have been no further clinical investigations of HSCs for back pain since this initial disappointing result although it is interesting to consider the potential contribution of HSCs in point of care bone marrow concentrate intradiscal injections(181-183).

1.7.9. Mesenchymal Stem Cells

Mesenchymal stem cells were originally described as marrow-derived, non-haematopoietic cells that form colonies of fibroblastic nature(184). MSCs have the potential to differentiate into multiple lineages including bone, adipose tissue and cartilage(184). Furthermore, MSCs have been demonstrated to have the capacity to differentiate into chondrocyte-like cells similar in phenotype to nucleus pulposus cells(185,186).

MSCs can be isolated from a variety of tissues: bone marrow, adipose tissue, cord blood, synovium(178,187). Important differences in cell differentiation potential and behaviour are observed among MSCs of different origins(188,189). Unlike ESCs, MSCs are non-tumorigenic and reported to lack the potential to undergo malignant transformation(190,191). Additionally, they demonstrate intermediate levels of MHC-I surface antigen expression, negligible levels of MHC-II surface antigen and immunoprivelege increasing their attractiveness for allogeneic applications (192). The International Society of Cellular Therapy defines MSCs by their characteristic plastic adherence in standard culture conditions, by cell marker expression of CD105, CD73, CD90 whilst lacking expression of CD45, CD34, CD14 or CD11b, CD 79a or CD19 and HLA-DR, and the ability to differentiate into osteoblast, adipocytes and chondrocytes in vitro(193).

The investigation of MSCs as a potential novel therapy for intervertebral disc degeneration was first reported by Sakai et al. in 2003(194). Since that time MSCs have been investigated in a variety of small to large animal models of disc degeneration (12,54,70,97,195-198). Implantation of bone marrow derived MSCs into various animal models of disc degeneration have been demonstrated to significantly increase disc height and MRI T2 signal (a marker of disc hydration), preserve histological structure and increase disc proteoglycan content(61,65,97). Bone marrow derived MSCs are the most commonly used stem cells in preclinical studies of intervertebral disc regeneration(199). Other MSC sources include adipose(39), synovium(198)and umbilical tissue. Autologous, allogeneic and xenogeneic MSCs have all been investigated for their potential to promote intervertebral disc regeneration.

Studies assessing the fate and viability of implanted MSCs have yielded variable results. Viability of implanted MSCs has been reported from 15 days to 48 weeks(200,201). However, other studies have reported leakage or non-viability of cells after administration. In a rabbit study of intervertebral disc regeneration in which MSCs were injected three weeks following intervertebral disc stab injury the authors found no evidence of regeneration or the persistence of cells at nine weeks following administration(202). The authors did, however, note the presence of osteophytes at the MSC injection site raising the possibility of MSC leakage contributing to osteophyte formation. In a similar vein, Omlor et al.(203) demonstrated loss of 90% of cells in three days following administration of MSCs to a nucleotomized porcine model of intervertebral disc degeneration. A recent study incorporating single-stage PET-MRI investigated the persistence of thymidine-kinase transfected magnetic iron oxide nanoparticle labelled MSCs in a canine model of disc degeneration(200). The authors demonstrated survival of MSCs, as assessed on PET scan, up to three weeks with no evidence of survival at four weeks.

There are various purported mechanisms of actions of MSCs. MSCs have been demonstrated in vitro to differentiate towards chondrocyte-like cells phenotypically similar to NP cells (185,186). In vivo studies demonstrating engraftment of MSCs and long term survival support this as one mechanism contributing to mechanism of action of MSC(201,204,205). Other authors have demonstrated the ability of MSCs to interact with NP cells and lead to the upregulation of proteoglycan synthesis(205,206). MSCs may also create a local milieu that promotes the regeneration of endogenous cells(207). Furthermore, mesenchymal stem cells demonstrate anti-inflammatory properties(208,209) and the ability to modulate the nucleus pulposus cell response to inflammatory cytokines(210).

1.7.9.1. *Preclinical Studies Comparing MSC and Chondrocyte Transplantation*

The few studies directly comparing chondrocytes and MSCs with regard to intervertebral disc

regeneration have produced mixed results. The study of Feng et. al.(211) comparing the regenerative potential of implanted autologous NPCs and MSCs in a rabbit model demonstrated comparable performance with regard to disc height and T2 signal preservation, proteoglycan production and collagen expression. In a porcine model comparing allogeneic non-disc derived juvenile chondrocytes (JC) and allogeneic MSCs Acosta et al. (68) demonstrated JC survival at 12 months with abundant cartilage formation at three months. Viable MSCs were not observed at any time-point and instead of cartilage only a collagen type I/type II enriched scar was observed. The conclusion of the authors was that the JCs were more appropriate for disc repair.

The use of chondrocytes in intervertebral disc regeneration presents specific challenges that may not be immediately evident. The majority of studies investigating the use of chondrocytes, of disc origin or otherwise, have focused on the use of autologous cells. Such cells, if of disc origin, must be harvested from the degenerate disc level, as to harvest from a healthy disc would increase the risk of subsequent degeneration of an otherwise healthy disc. These cells may already possess impaired function given their origin from a degenerate disc and thus potentially reduced efficacy in promoting disc regeneration(212). Furthermore, harvested cells require cell culture under GMP (Good Manufacturing Practice) conditions in facilities that come at significant expense and may not be available in many centres. Harvest of autologous cells from a non-disc source requires a separate operation, with potential morbidity, while still necessitating cell culture expansion. Allogeneic chondrocytes have been investigated in a rabbit model with demonstrated success(213) but have yet to be trialed in the clinical setting.

1.7.9.2. Clinical Trials of MSCs

Given the challenges associated with the application of chondrocytes in the treatment of intervertebral disc degeneration it is not surprising that many reports of cellular therapy for intervertebral disc degeneration focus on the use of mesenchymal stem cells.

To date there have been four published reports investigating mesenchymal cells for the treatment of intervertebral disc regeneration with an additional nine studies listed on clinicaltrials.gov(214).

The first clinical report of MSC therapy for intervertebral disc regeneration was of two patients who received autologous expanded MSCs via endoscopic placement in the nucleus pulposus in 2005(215). Both patients were diagnosed to have intervertebral disc degeneration via a combination of clinical examination, MRI and plain radiography. Patients received percutaneous administration of autologous culture expanded MSC in collagen sponges. Both patients reported significant reductions in back pain, Japanese Orthopaedic Association scores improved and MRI T2 signal was improved at the treated discs.

In 2011, Orozco et al.(216) reported a non-controlled pilot study of 10 patients with chronic back pain and degenerative disc disease treated with autologous culture expanded bone marrow derived mesenchymal stem cells percutaneously injected into the nucleus pulposus. Pain and disability were significantly reduced by three months with persistent improvement continuing throughout the 12 months of the study. MRI demonstrated increased disc hydration at 12 months on T2 imaging.

In the follow-up to the 2011 study of Orozco et al.(216) Noriega et al.(217) recently reported the results of a randomised clinical trial of allogeneic bone marrow mesenchymal stromal cells involving 24 patients with chronic back pain and degenerative disc disease. Half of the patients received percutaneous administration of 25×10^6 marrow stromal cells. Patients receiving MSC therapy demonstrated a statistically significant improvement in pain and disability that peaked at three months and was largely maintained to 12 months. Patients who received MSCs also demonstrated a statistically significant improvement in MRI Pfirrmann Grades in the treated discs. This report is the first published describing the clinical application of allogeneic MSCs for disc degeneration.

Another uncontrolled clinical study investigated autologous cultured expanded bone marrow derived MSCs(218) for the treatment of degenerative disc disease with radicular symptoms in 33 patients. Patients in this trial received an average cell dose of 2.3×10^7 MSCs. Numerical pain score change scores relative to baseline were significantly reduced at most time-points up to 72 months. Functional rating index scores also demonstrated significant improvement beyond the minimum clinically important difference at all time points except 12 months. Additionally, of the 20 patients who underwent post-operative MRI 85% demonstrated a reduction in disc bulge size.

A variant of MSC therapy for intervertebral disc degeneration is the use of autologous bone marrow concentrate as described by Pettine et al.(181,183,219). This approach entailed bone marrow aspiration of approximately 55ml with point of care processing via a bone marrow concentration system to produce ~6ml of bone marrow concentrate (BMC) for injection. The BMC was found to have a total nucleated cell concentration of approximately 121×10^6 per ml of which 0.0025% (~2700 cells per ml) were colony-forming unit-fibroblasts (CFU-F), which the authors consider equivalent to MSCs, and 1.66×10^6 cells per ml were CD34+ lineage cells (haematopoietic-endothelial progenitor cells). The authors demonstrated that over three years, only six of the 26 patients who received BMC therapy progressed to surgical intervention with the remaining 20 patients demonstrating significant VAS and ODI improvements. Furthermore, eight of 20 patients with post-treatment MRI imaging demonstrated at least one grade increase in modified Pfirrmann grade at one year. Given the relatively small number of CFU-F cells administered to each patient in this trial, approximately 16,000 cells, compared to the number administered in the MSC studies above (~10 – 20 x 10^6 cells) it is difficult to conclude the beneficial effects observed are secondary to the MSCs alone. Indeed, the patients in the clinical trials of Pettine

et al. received almost 1000 times as many CD34+ lineage cells as MSCs. However, this trial does present an interesting variation of autologous bone marrow derived cell therapy that obviates the need for expensive GMP facilities.

The use of umbilical MSCs for the treatment of intervertebral disc degeneration was reported for two patients in China(220). The patients, both suffering lower back pain with concordant MRI and discography findings, received 1×10^7 umbilical MSCs via percutaneous injection. Both patients reported significant improvement in VAS pain and ODI scores over 24 months with one patient demonstrating increased MRI T2 signal intensity in the treated disc.

Although only small studies of MSC therapy for intervertebral disc degeneration have been reported to date larger studies are underway at present. Clinicaltrials.gov lists twenty three studies of stem cell therapy for intervertebral disc degeneration, nine of which are of mesenchymal stem cells for intervertebral disc regeneration with cell sources including autologous bone marrow, adipose tissue and degenerate intervertebral discs and allogeneic bone marrow(221). To date, the largest clinical trials are the Mesoblast sponsored trials using mesenchymal progenitor cells for disc regeneration, which will be discussed below.

1.7.9.3. Mesenchymal Progenitor Cells

Mesenchymal progenitor cells (MPCs) are a subpopulation of MSCs that can be isolated using magnetic cell sorting and antibodies and subsequently expanded in culture to produce a homogeneous population of cells(179,222) in distinction to the mixture of cell types found in bone marrow isolates mentioned above. These cells have extensive proliferative capacity, are the major colony forming population present in the bone marrow, and have greater plasticity than the mature stromal cells(179,222,223). Furthermore, the cells can be used in an allogeneic capacity due to minimal immunogenicity and demonstrated in vivo allogeneic tolerance(192,224). This enables the development of MPCs as an "off the shelf" therapeutic, with batch to batch consistency. The major advantages of such an approach include the avoidance of additional procedures for harvesting cells and obviation of the necessity of cell culture expansion facilities at numerous institutions.

A previous study by our group utilized immune-selected STRO-3+ MPCs, prepared from ovine bone marrow aspirates, to investigate the reconstitution of lumbar discs in an ovine model of disc degeneration, mediated by injection of the enzyme Chondroitinase-ABC into the NP (196). This study demonstrated that MPCs were effective in supporting recovery of disc proteoglycans and disc height index. Subsequent in-vitro(225) and in-vivo(96) studies demonstrated that combination of MPC with the pharmaceutical agent, Pentosan Polysulfate (PPS), not only enhanced their proliferation and chondrogenic differentiation but also suppressed osteogenesis. Using an ovine model of lumbar disc

degeneration, induced by the surgical creation of a horizontal lesion in the periphery of their AFs, it was reported(12) that six months after the injection of a formulation of MPCs with PPS into the NP of degenerate discs, increased disc height, reduced morphological evidence of matrix failure and significantly elevated PG content of their NP relative to controls was observed.

The studies above demonstrate the potential of MPC therapy to promote intervertebral disc regeneration in the context of a preclinical model of intervertebral disc degeneration. Neither of these studies, however, adequately replicate the post lumbar discectomy intervertebral disc in which annular and nuclear defects are present in addition to disc degeneration. The presence of a marked annular defect poses particular challenges given the potentially for biological therapies to leak out of the incompetent annulus(202). Furthermore, the post discectomy intervertebral disc also displays a defect in the nucleus pulposus. This combination is associated with a reduction in disc height, reduced proteoglycan content and MRI T2 signal(11). Thus, it is important to explore the potential of these cells to promote intervertebral disc regeneration in the context of a model of the post lumbar discectomy intervertebral disc.

The same ovine formulation of MPCs and PPS used in the ovine disc degeneration model was also evaluated in an ovine model of microdiscectomy(11). In this pilot study(11), a suspension of allogeneic MPCs with PPS was applied to a gelatin sponge scaffold placed in a full depth defect created in the AF of ovine lumbar discs then closed with fibrin glue. The two adjacent lumbar discs were used as untreated controls. Following euthanasia six months later, it was observed that the microdiscectomy defects implanted with the MPC+PPS and gelatin sponge exhibited significantly more new matrix regeneration and preservation of disc height compared to defects that were untreated or received the scaffold alone(11).

Although the pilot study provided important information in regard to a potential surgical adjunct to support disc repair following microdiscectomy, since PPS was known to promote regeneration of cartilage in osteoarthritic joints(226) it was unclear whether the MPC or PPS were the active mediators of the repair observed in this model or whether both components acted synergistically. To resolve this question, an in-vitro study was undertaken to identify the mechanism of action of combining PPS with MPCs(227). The results of this study indicated that the activation of MPC by PPS in culture was mediated by its migration to nucleus of the MPCs, where it upregulated specific gene pathways responsible for cell replication, differentiation and biosynthesis of proteoglycans(227). Significantly, these studies also demonstrated that after culturing the MPCs with PPS for 24 -48 hours, then eliminating the presence of PPS from the cultures, the MPCs retained their genetic re-programing, i.e. they were now "primed" to differentiate to a chondrogenic phenotype, and as such should be superior to MPCs alone in supporting repair of injured cartilaginous tissues(227).

In addition to the above preclinical work human allogeneic MPCs administered percutaneously to affected intervertebral discs in patients with single level degenerative disc disease and lower back pain have been demonstrated to produce significant reductions in pain scores and analgesic requirements in a successful Phase II Clinical Trial(224). The primary composite endpoint of this trial, consisting of a 50% reduction of VAS low back pain, 15 point reduction in Oswestry Disability Index and no additional intervention, was achieved by 41% of patients who received 6×10^6 MPCs compared to only 13% of those receiving saline control(228). This effect persisted to three years for 38% of the 6×10^6 MPCs group and 10% of the saline control group(228). This trial has been followed by an ongoing Phase III clinical trial that is still enrolling patients as of late 2017.

Percutaneous injection of MPCs, however, is targeted for back pain and does not represent the ideal therapy for patients with radiculopathy undergoing microdiscectomy. The microdiscectomy operation is performed to remove a portion of herniated disc causing symptoms by neural compression. At the completion of the operation the patient is left with a defect in the annulus fibrosus through which the offending disc fragment has herniated. The annular defect will thus serve as the route of administration of the cellular therapy. However, as noted in previous preclinical trials, a significant challenge in administration of cellular therapy to intervertebral discs is ensuring retention of the cellular material(229). Various carriers have been used in previous studies including fibrin(68), collagen(215), and hydrogel carriers(61).

1.8. Chondrogenesis and the Chondrogenic Differentiation of Mesenchymal Stem Cells

Promotion of chondrogenesis within the degenerate intervertebral disc is one of the keys to regenerative therapy. Chondrogenic differentiation of MSC or MPC, in vitro, is generally achieved by exposing these cells to growth factors such as TGF-Beta, IGF-1, BMPs, alone or in combination(230,231). However, there are inherent problems associated with using these proteins in vivo. Firstly, they have short biological half-lives, thus potentially requiring the administration of multiple doses. But more importantly, their clinical application has been reported to be associated with serious side effects(232-235). The potential difficulties associated with other known promoters of chondrogenic differentiation of MSCs increases the attractiveness of PPS as a chondrogenic agent.

1.9. Conclusion

Disc degeneration and low back pain are a major cause of morbidity and disability worldwide. Lumbar disc herniation is a relatively common presentation of degenerative disc disease. Lumbar microdiscectomy surgery successfully treats radicular symptoms in the vast majority of patients but fails to address the underlying pathophysiology of intervertebral disc degeneration. Lumbar fusion and total

disc arthroplasty are surgical procedures often used to treat discogenic back pain, however, their application in this context remains contentious with some studies showing equivalence to conservative management(134,136). Furthermore, lumbar fusion may be complicated by pseudoarthrosis(133) or adjacent segment disease(139). Total disc arthroplasty has yet to demonstrate superiority to fusion in long-term outcomes(141,150,236). As such fusion and total disc arthroplasty remain invasive, destructive, sub-optimal therapies that fail to address the underlying pathophysiology of degenerative disc disease.

Given the failure of conventional medical and surgical therapy to address the underlying pathology of intervertebral disc degeneration there have been sustained efforts to develop novel therapies to arrest and reverse this condition. Our group was instrumental in the development of mesenchymal progenitor cells as a novel therapy for disc degeneration. The percutaneous administration of mesenchymal progenitor cells in the context of discogenic low back pain has demonstrated positive results in Phase II studies(224) and has led to a Phase III clinical trial under way at the time of submission of this thesis. However, percutaneous administration of MPCs may not represent the ideal therapy in the context of the post lumbar discectomy intervertebral disc.

We propose that administration of MPCs primed with PPS at the time of lumbar discectomy may provide a method of successfully treating disc degeneration and reducing the incidence of low back pain and its associated disability. The aim is to reduce reherniation and further surgery that unfortunately befall many patients who undergo lumbar discectomy. The results of the pilot study of MPCs combined with PPS in an ovine model of lumbar discectomy(11) have demonstrated the promise of MPC therapy in the post lumbar discectomy intervertebral disc, however, further larger studies are required.

In order to adequately test this hypothesis it is important to use an appropriate preclinical model. There is a relative paucity of large animal models of the post lumbar discectomy intervertebral disc. As detailed previously the ovine model has many of the ideal characteristics of an animal model of intervertebral disc disease including the lack of notochordal cells in the adult intervertebral disc(93), the similarities in size to the human disc and spine biomechanics(92), despite the quadrupedal nature of sheep. In order to adequately replicate the post lumbar discectomy disc in an animal model it is important to choose an appropriate method of creating a similar environment. The drill bit injury(42) and lumbar discectomy models(11) are attractive in this regard as they both consistently produce a full depth annular injury with extension into the nucleus. Of the preclinical models described these two represent the most promising methods of replicating the post lumbar discectomy intervertebral disc. As such a direct comparison of these two models in the sheep over a six month period is described in Chapter 3, the first experimental chapter.

Following selection of an appropriate preclinical model of the post lumbar discectomy intervertebral disc this model will be used to investigate the potential of MPC primed with PPS in a gelatin scaffold with fibrin glue to promote intervertebral disc regeneration following lumbar discectomy. As detailed previously, the MPC and PPS combination was demonstrated to promote intervertebral disc regeneration in an ovine model of lumbar discectomy in a pilot study. However, it was not known if the PPS or MPC were producing the beneficial effects. Furthermore, PPS priming of MPCs in vitro has been demonstrated to promote persisting changes in MPCs conducive to promoting intervertebral disc regeneration, i.e. cellular replication, chondrogenic differentiation and proteoglycan production(227). In order to determine if PPS priming leads to persisting changes in MPC phenotype in vivo and if this is advantageous in promoting intervertebral disc regeneration PPS primed MPCs will be compared directly to unprimed MPCs, in an ovine model of the post lumbar discectomy intervertebral disc, in Chapter 4. The priming of MPC with PPS prior to administration into the intervertebral disc also has the potential regulatory advantage of representing a manufacturing process rather than co-administration. This regulatory advantage may ultimately ease the translation of this novel approach to clinical practice.

Optimization of the discectomy procedure extends beyond the introduction of regenerative therapy at the time of lumbar discectomy. As detailed above, despite being the most commonly performed spine surgery procedure worldwide, a significant lack of clarity remains with regard to appropriate post-operative care and the role, if any, of post-operative activity restrictions. A variety of international surveys to date have demonstrated the heterogeneity of post-operative care practice(120,237-239). In order to gain an accurate assessment of the lumbar discectomy peri-operative care practices of Australasian neurosurgeons a survey was conducted with the support of the Neurosurgical Society of Australasia and is reported in Chapter 5. This is the first survey conducted of Australasian Neurosurgical peri-operative care practices and provides an up to date assessment and demonstrates the heterogeneity present in such practices within even the relatively small community of Australasian neurosurgeons.

After demonstrating the heterogeneity present in post-operative care following lumbar discectomy within Australasian neurosurgical practice this thesis will attempt to investigate the role, if any, of post-operative activity restrictions following lumbar discectomy in contemporary practice. The studies performed to date attempting to address this questions were either not randomised(125,126) or under powered and subject to the vagaries of self-report(127) when determining patient activity status. In the final experimental chapter the protocol of an appropriately powered randomised controlled trial investigating the role of activity restrictions in post-operative care following lumbar discectomy surgery will be described. This study benefits from the use of a wearable activity tracker that measures patient

posture and activity enabling accurate correlation of patient activity during the post-operative period to outcome over the study length. An interim analysis of this study, now in its second year at the time of submission of this thesis will also be presented.

Thus, this thesis will attempt to re-engineer lumbar discectomy surgery via a combination of preclinical and clinical approaches. The preclinical component consists of the development of an appropriate large animal model of the post lumbar discectomy disc to facilitate preclinical investigation and development of a novel cellular therapy to be administered at the time of lumbar discectomy to reverse the pathological process of disc degeneration. The clinical portion is comprised of characterization of the current lumbar discectomy peri-operative care practices of Australasian Neurosurgeons and investigation of the role, if any, of post-operative activity restrictions following lumbar microdiscectomy via a randomised controlled trial.

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Review Article

A Review of Animal Models of Intervertebral Disc Degeneration: Pathophysiology, Regeneration, and Translation to the Clinic

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Lower back pain is the leading cause of disability worldwide. Discogenic pain secondary to intervertebral disc degeneration is a significant cause of low back pain. Disc degeneration is a complex multifactorial process. Animal models are essential to furthering understanding of the degenerative process and testing potential therapies. The adult human lumbar intervertebral disc is characterized by the loss of notochordal cells, relatively large size, essentially avascular nature, and exposure to biomechanical stresses influenced by bipedalism. Animal models are compared with regard to the above characteristics. Numerous methods of inducing disc degeneration are reported. Broadly these can be considered under the categories of spontaneous degeneration, mechanical and structural models. The purpose of such animal models is to further our understanding and, ultimately, improve treatment of disc degeneration. The role of animal models of disc degeneration in translational research leading to clinical trials of novel cellular therapies is explored.

1. Introduction

Lower back pain causes more global disability than any other condition worldwide [1] and is an enormous financial burden due to costs related to loss in working hours as well as for its medical treatment. Up to 80% of people may experience lower back pain at some stage in their life, with prevalence ranging from 15 to 45%. Chronic lower back pain can be caused by degenerative lumbar disc disease which produces discogenic pain [2]. This needs to be distinguished from radicular pain, which is pain resulting from nerve root compression, often due to a disc prolapse. Lumbar disc degeneration is a complex process manifested by changes in cellular, matrix, endplate, and the neurovascular components of the intervertebral disc. Given the significant contribution of disc degeneration to the enormous disease burden of lower back pain numerous animal models have been developed in an effort to further understanding and treatment of this condition. In order to compare and contrast the merits of different models a basic appreciation of the structure of the intervertebral disc and underlying pathophysiology is a prerequisite.

2. The Intervertebral Disc

The intervertebral disc is a complex multicomponent structural tissue consisting of an outer fibrous ring, the annulus fibrosus (AF), and an inner hydrated gel-like substance, the nucleus pulposus (NP) [3]. It is the largest avascular structure in the body. Nutrition of the intervertebral disc is provided by diffusion through the cartilaginous endplates (CEP). The CEP are specialized interfaces that connect the intervertebral disc with the adjacent vertebral bodies. The AF is a fibrocartilaginous tissue rich in type I and II collagen and assembled as lamellae fibres oriented at varying degrees to adjacent lamella in different locations and species. The AF connects the caudal and cranial vertebral bodies of the spinal

column [4]. The main cell types of the AF are fibroblasts that synthesize not only the lamellar collagen, but also proteoglycan (PGs), elastin, and other noncollagenous proteins [5]. The tough fibrous composite structure of the AF encapsulates the gelatinous NP and provides the necessary mechanical strength and resilience to allow the disc to recover from deformation arising from axial, rotational, and bending loading. In healthy discs the NP consists of a hydrated gel composed of predominantly type II collagen and large amounts of PGs. Aggrecan is the most abundant PG type in the NP. Due to its high anionic charge aggrecan attracts and retains high levels of water molecules within the NP thereby maintaining a high hydrostatic swelling pressure that confers resistance to disc deformation and maintenance of disc height [3, 6].

Cells of the disc NP are derived from the notochord. In man these cells are retained throughout childhood but with maturity disappear and are replaced by chondrocyte-like cells [5]. The loss of notochordal cells from the NP represents an important early step along the path to degenerative disc disease and in this regard it should be noted that apart from a limited number of animal species (chondrodystrophoid dogs, old sheep, and cattle) NP notochord cells are retained throughout their life.

3. Intervertebral Disc Degeneration

Human intervertebral disc degeneration is a complex and incompletely understood multifactorial process with contributions from genes, mechanical stresses, cellular senescence, and alterations in nutrition via the limited vascular supply [7]. With respect to mechanically loading the intervertebral disc there is a delicate balance between "normal" mechanical loading, which is required for maintenance of an optimal disc cellular phenotype [8, 9], and excessive mechanical loading that causes damage. Excessive loading can result from excessive bodyweight [10] or trauma and produces many of the features of degeneration that can be visualized by histological and radiological methods.

Studies comparing degenerate discs with nondegenerate controls have demonstrated increased evidence of senescent cells in degenerate intervertebral discs [11]. Such cells lose the ability to divide, thus potentially contributing to the decreased cellularity of the diseased degenerate intervertebral discs. Moreover, the senescent cells have a reduced ability to function. Thus they produce less matrix which, in turn, further compromises the structure of the intervertebral discs.

Intervertebral discs comprise the largest essentially avascular tissue in the human body. Only the outermost layers of the AF contain blood vessels. The cells of NP are dependent on diffusion of nutrients from capillary buds in the cartilaginous endplate to meet their metabolic needs [12]. The cells in the NP are therefore metabolically compromised by this limited vascular and nutritional supply and may promulgate intervertebral disc degeneration. Causes of impaired nutrition to the intervertebral disc include endplate calcification, microvascular disease, and smoking and have all been associated with early disc degeneration.

Ultimately there is an imbalance between the rates of production and breakdown of the matrix components leading
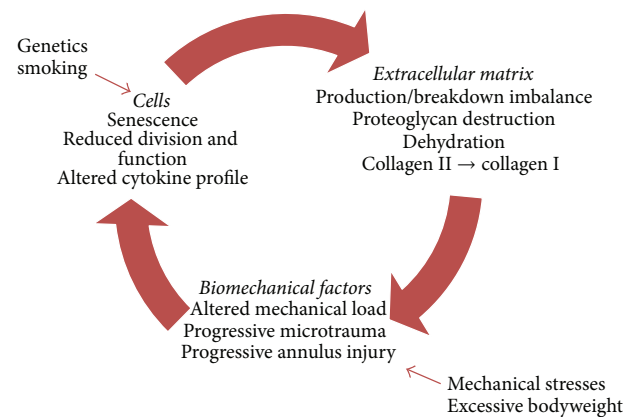

FIGURE 1: Schematic of the process of disc degeneration demonstrating multifactorial pathophysiology and interplay of cellular, matrix, and biomechanical factors. Modification of figure from Vergroesen et al. [17].

to a cascade of events (see Figure 1) consisting of alterations in matrix synthesis, reduced aggrecan synthesis, and a transition from type II to type I collagen production [13], reduction in cellular viability and activity, and alterations in cytokine profile upregulating the breakdown of proteoglycans, all leading to dehydration and loss of mechanical integrity of the intervertebral disc [6, 12]. The dehydration of the intervertebral disc reduces the mechanical support provided by the swelling pressure of the previously hydrated NP. This alters the mechanical load to which the AF is exposed and thus the tension in the AF collagen fibres. This leads to subsequent progressive microtrauma of these fibres [6]. The degeneration of the AF and subsequent tears in this structure predispose patients to disc herniation, wherein fragments of disc tissue herniate through this annular defect causing neural compression and radicular pain [14]. As the mechanical and structural integrity of the disc progressively deteriorates neurovascular invasion may occur via annular tears. Neurovascular invasion extending to the NP via annular fissures has been demonstrated in painful discs in clinical studies [15]. In contrast, control (nonpainful) discs demonstrated restriction of vascular and neural supply to the outer annulus [15]. This process of neoinnervation of the degenerate intervertebral discs is hypothesized to be a significant contributor to the development of back pain [16].

3.1. Animal Models of Disc Degeneration. The development of appropriate animal models of intervertebral disc disease is necessary to gain insight into the pathophysiology and to develop and test potential therapies. In vitro and in silico (computer based) systems can be helpful to investigate specific components of intervertebral disc degeneration. However, given the complexity inherent in the intervertebral disc with biochemical, biomechanical, nutritional, and metabolic factors acting simultaneously, in vivo animals are able to more faithfully replicate this environment. A range of animal models and mechanisms of replicating the process of degeneration have been investigated and utilized in efforts to

develop appropriate models of intervertebral disc degeneration. However, given the extreme complexity of this system no perfect model currently exists.

Initial discussion will focus on the merits and inadequacies of particular species as models for human intervertebral disc degeneration. This will be followed by a discussion of the comparative merits of the various methods of inducing disc degeneration.

3.1.1. Comparison of Various Animal Models. Animal models range from small rodents such as mice knockout models [18] to rats [19], rabbits [20], dogs [21], goats [22], sheep [23], and primates [24, 25]. Various mechanisms of inducing degeneration have been described for these animal models which are summarized in Table 1. When considering the potential suitability of an animal model several important characteristics must be taken into account and these are discussed in Table 1.

3.1.2. Persistence of Notochordal Cells. The vertebral column and thus intervertebral discs of all mammals arise from aggregation of the mesenchyme around the notochord and subsequent segmentation during development [26]. Notochordal cells persist in the NP of the majority of species (e.g., mice, rats, rabbits, and pigs) into adulthood. However, the number of these cells decreases rapidly following birth in humans and notochordal cells are completely absent from the NP by early adulthood [26]. Sheep and goats are among the few animals to also lose the notochordal cells rapidly from the NP following birth. Dogs are divided into two populations with regard to notochordal persistence into adulthood. Chondrodystrophoid (CD) dogs rapidly lose the notochordal cells following birth and as such are predisposed to intervertebral disc degeneration in later life. Nonchondrodystrophoid (NCD) dogs have persistent notochordal cells and are far less inclined to disc degeneration. The persistence of notochordal cells is an important consideration as these cells have a significant influence on the intervertebral disc by influencing proteoglycan metabolism [27, 28], hyaluronan production [29], and possible progenitor cell function [26].

Loss of intervertebral disc notochordal cells may be observed in animal models with otherwise persistent notochordal cells following adequate stimulus [30, 31]. Apoptotic processes have been demonstrated to play a significant role in this process of notochordal cells loss [31, 32] and are also observed in human aged and degenerate discs [33]. Thus such animal models may have greater relevance following the loss of notochordal cells than otherwise.

However, given the use of animal models to investigate cellular regenerative therapies for the treatment of disc degeneration the potential presence of a preexisting precursor cell population may complicate investigation of the regenerative potential of such therapies. For instance, in cell transplantation therapies, one cannot be sure that it is not the resident notochordal cells which are responsible for the regenerative effects, instead of, or in combination with, the transplanted cells.

3.1.3. Disc Size and Geometry. Intervertebral discs vary markedly across species and according to location within the spine.

The discs of most animal models are smaller than human intervertebral discs. Disc size affects solute diffusion in the intervertebral disc. Given the largely avascular nature of the intervertebral disc and dependence on diffusion to meet nutritional requirements this is of particular significance to the clinical relevance of animal models. Given the size discrepancies between common animal models and humans investigators have analyzed disc geometry hoping to better determine the relevance of particular models to the human intervertebral disc. In a study by O'Connell et al. [34] the geometries of intervertebral discs of commonly used animal models were analyzed with regard to their similarity to the human intervertebral discs as measured by relative proportions (e.g., disc height, width, and NP size). Interestingly, the authors ranked mouse lumbar intervertebral disc as the animal model most geometrically analogous to the human intervertebral disc.

3.1.4. Disc Mechanical Forces. The vast majority of animal models of intervertebral disc disease are quadrupedal. The only bipedal models available, certain primates to varying extents (e.g., rhesus monkey [35]) and the bipedal mouse and rat [36], present ethical dilemmas that preclude their usage in most institutions. Given that the mechanical loading to which human intervertebral lumbar discs are exposed is significantly influenced by the upright posture it may be thought that this precludes usage of quadrupedal animal models. However, muscle contraction and ligament tension is a significant contributor to the load to which intervertebral discs are exposed [37]. It has been hypothesized that the load exerted on the lumbar intervertebral discs of large animals by these structures may be even greater than that observed in humans resulting from the bipedal stance due to the increased complexity of stabilizing a horizontally aligned spine versus a vertically balanced spine [26].

3.2. Animal Models. Taking the above general considerations into account the following models are those most commonly described for use as in vivo models of intervertebral disc degeneration.

3.2.1. Rodent Models. Mice and rat models, despite the obvious difference in intervertebral disc size, have significant advantages with regard to ease of use and application of technology. Genetic knockout and mutation mice models have enabled the investigation of the effects of the elimination of particular proteins, for example, collagen II [18], on disc function. Bipedal mouse and rat models were created through bilateral mid-humeral surgical and tail amputations [36]. Bipedal mice were observed to demonstrate accelerated NP degeneration with frequent NP herniation [40]. However, more recent studies have indicated that bipedal rats do not assume a more erect posture than their quadrupedal peers [69] calling into question the cause of the observed increased disc degeneration.

The mouse and rat tail provide a readily accessible model for intervertebral disc degeneration through mechanical injury, asymmetrical compression, or administration of digestive enzymes [39, 70].

TABLE 1: Continued.

Animal	Notochordal cells in adult intervertebral disc	Mechanism	References
Pig	Present	*Structural* Nucleotomy	Acosta et al. [61]
Sheep	Absent	*Structural* Partial thickness annulotomy	Osti et al. [62], Oehme et al. [63], Melrose et al. [64]
		Annular incision and partial nucleotomy (simulated microdiscectomy)	Oehme et al. [23]
		Chondroitinase ABC	Ghosh et al. [65]
Macaque	Present	*Spontaneous* Age related degeneration	Nuckley et al. [66]
Baboon	Present	*Spontaneous* Age related degeneration	Lauerman et al. [24] and Platenberg et al. [25]
Rhesus monkey	Present	*Structural* Annulotomy $\pm$ collagenase	Stern and Coulson [67]
		Bleomycin injection of subchondral bone	Wei et al. [68]

NP indicates nucleus pulposus.

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However significant limitations exist for such models:

(1) Persistent notochordal cells: limiting the potential relevance of such models to the clinical environment particularly with regard to testing potential therapeutics.

(2) Differing mechanical loading: rodent tail models: the tail may have significantly different mechanical loading to the human lumbar spine although this has been disputed by some authors.

(3) Significantly smaller disc size reducing the nutritional challenge.

(4) Ethical concerns regarding the bipedal mouse.

3.2.2. Rabbit Models. The rabbit model of intervertebral disc degeneration has been utilized by several authors for investigation of disc degeneration and of potential therapeutic agents [20, 71]. Major advantages of this model are the higher degree of homology to the human intervertebral disc with the presence of facet joints and paravertebral muscles and ligaments in comparison to the rodent tail models [51], the larger size of the animal and intervertebral discs, and the cost-effectiveness as a model relative to large animals. Limitations relate to the persistence of notochordal cells and the significant variation from human geometry [72].

3.2.3. Canine Models. As discussed previously CD dogs demonstrate a decrease of notochordal cells from birth onwards with complete loss by early adulthood predisposing the animal to intervertebral disc degeneration. The CD dogs, among which beagles and dachshunds number, are well-characterized models of spontaneous intervertebral disc degeneration [57]. The larger size of the disc space relative to rodents makes administration of intradiscal therapeutic agents technically less challenging [58]. Similarities exist with regard to the gross pathology, histopathology, and glycosaminoglycan content among humans and canines in intervertebral disc disease [21]. Differences exist with regard to the thicker cartilaginous endplates in humans, the presence of growth plates within the vertebrae of the canine [21], and intervertebral disc size. Additional ethical concerns exist with regard to the use of dogs for experimental research in many parts of the world.

3.2.4. Goat Models. Goats have previously been used as models for intervertebral disc degeneration [22, 60]. Advantages of the use of this species include anatomical similarities with regard to size and shape with respect to the human intervertebral disc, comparable mechanical load [73], absence of notochordal cells in the adult [74], and the pragmatic benefits of a hardy, economical animal model that tolerates surgery well [22].

3.2.5. Sheep Models. The sheep model has proven to have particular merit for several major reasons. Firstly, the sheep, similar to humans, suffers from a loss of notochordal cells in early adulthood, predisposing the sheep intervertebral discs to degeneration [75]. The sheep is of a roughly similar size to humans and, despite its quadrupedal stature, is exposed to very similar mechanical stresses to the human intervertebral disc [76]. The ovine spine has previously been used to model disc degeneration [62–64] and test implant devices and in the preclinical investigation of cellular therapies [23, 77–79]. Similar to the goat the sheep is a hardy animal with demonstrated ability to tolerate surgical intervention.

3.2.6. Porcine Models. Porcine models have been utilized in models of intervertebral disc degeneration and in the preclinical assessment of biological therapies such as mesenchymal stem cells [61, 80]. Major advantages attributed to the porcine model include the similarity in size of the disc to the human intervertebral disc and overall size of the animal. However, this advantage is significantly offset by the persistence of notochordal cells into adult life in the porcine model [80], potentially confounding interpretation of investigations of disc degeneration and regenerative therapies.

3.2.7. Primate Models. Spontaneous disc degeneration has been demonstrated in baboon and macaque models [24, 25, 66]. Both species are quadrupedal for locomotion but spend large amounts of time in semierect and erect positions. Rhesus monkeys have also been used as models of disc degeneration following annulotomy ± intradiscal administration of collagenase [67] and subchondral administration of bleomycin [68]. The advantages of such nonhuman primate animal models include intervertebral disc size closer to humans, comparable anatomy, spontaneous disc degeneration, and exposure to mechanical stresses compatible with erect posture. However major ethical and practical considerations (e.g., expense and housing) are associated with the use of nonhuman primate animal models significantly restricting their use for such studies in many institutions.

3.3. Comparison of Mechanisms. Given the complexity of human disc degeneration no animal model can perfectly mimic the entire pathophysiological process. Disc degeneration in animal models is typically initiated by various chemical (e.g., chondroitinase ABC injection [81]) or mechanical (e.g., surgical incision [63], nucleotomy-NP aspiration [82], and drill bit injury [22]) stimuli though it can develop spontaneously in some animals [83].

3.3.1. Spontaneous Models. Spontaneous disc degeneration occurs in a limited number of species, with inconsistent onset and development over a long time frame. The most well-studied species with regard to spontaneous disc degeneration are the sand rat and the chondrodystrophoid dog species. As described above spontaneous disc degeneration has also been observed in nonhuman primates.

Sand Rat. The sand rat is native to east Mediterranean deserts [84] and was first described to undergo spontaneous disc degeneration by Silberberg et al. [48]. When fed a standard laboratory diet the sand rat develops diabetes and widespread disc degeneration [85]. The degenerative changes consist of loss of notochordal cells, annular disorganization, cellular

metaplasia, endplate sclerosis, and the formation of peripheral osteophytes [50]. In a longitudinal study conducted by Gruber et al. [49] radiographic evidence of degeneration was evident by two months with older animals demonstrating disc space wedging, narrowing, irregular disc margins, and endplate calcification. The degenerative process commenced in the NP with subsequent degeneration of the facets and endplates occurring only after disc herniation had developed [48]. Additionally the sand rat has been successfully used in studies of cellular therapy of disc degeneration despite the significant technical challenges this entailed [86].

Genetically Modified Mice. Genetically modified mice models have been developed to investigate the contribution of specific proteins to disc degeneration. Genetically modified mice with collagen IX mutations demonstrated increased cervical degeneration [38]. Similarly mice with collagen II mutations underwent premature endplate calcification and subsequent disc degeneration [18].

Canines. As detailed previously CD dogs demonstrate loss of notochordal cells from birth onwards and progress to demonstrate gross pathology and histopathological and glycosaminoglycan content changes similar to humans in intervertebral disc degeneration [21]. There are also marked similarities between magnetic resonance images of intervertebral disc degeneration in different stages of progression in canines and humans [87, 88]. Canines (both CD and NCD) also undergo routine clinical treatment for degenerative disc disease including decompressive surgery [21]. The chondrodystrophoid dog has long served as an animal model of intervertebral disc degeneration and will continue to do so into the future.

Primates. Baboons and macaques have both been demonstrated to undergo spontaneous disc degeneration [25, 66]. As nonhuman primates that spend a significant proportion of time in erect and semierect postures such animal models demonstrate significant potential for modeling human disc degeneration. However, ethical and pragmatic consideration will likely limit their usage.

Spontaneous models of disc disease can be particularly useful in providing models of disc degeneration. However, the long and at times unpredictable course of spontaneous degeneration often limits their utilization in studies of potential therapies.

3.3.2. Mechanical Animal Models of Disc Degeneration. Mechanical models afford the advantage of initiating the degenerative cascade at a defined time point in a replicable fashion. Epidemiological studies have suggested the association between exposure of the spine to force and disc degeneration [89]. Mechanical models of disc degeneration can be broadly divided into two groups: compression and instability, although there is overlap between the two groups [83].

Compression. Compression involves the application of altered mechanical stresses to the intervertebral disc through

mechanism such as bending [43], postural change [90] (i.e., the bipedal rat), or cyclical compression [45].

(1) Bending. Bending of the rat tail is one of the earliest reported methods of inducing disc degeneration [43]. In pioneering studies by Lindblom [43] rat tails fixed into bent shapes demonstrated annulus degeneration on the concave side with connective tissue injury and reduced cellularity. In a more recent study Court et al. [39] were able to demonstrate increased cell death and decreased aggrecan gene expression in the concave side of a disc compressed by forceful fixed mouse tail bending. Such differences were not observed in mice tails exposed to only slight bending.

(2) Postural Change. The bipedal rat and mouse models, as described above, are based on the hypothesis that surgically modified animals will spend more time in an erect posture thus exposing the intervertebral discs to increased mechanicals stress. Given the more recent findings indicating bipedal rats do not spend an increased time in an erect posture and, in fact, possibly less time than their quadrupedal counterparts [69] it is interesting to reflect as to the aetiology of the underlying degenerative changes observed in the primary studies.

(3) Chronic and Cyclical Compression. Researchers have investigated the application of static and cyclical compression to the intervertebral disc. Iatridis et al. [44] described a rat tail compression model to apply chronic compression. This consisted of an Ilizarov-type apparatus (an external fixation device enabling application of mechanical force across the intervertebral disc) applied to the tail of rats. Rats were assigned to sham, immobilization, or compression groups. The immobilization and compression groups demonstrated decreased disc thickness, axial compliance, and angular laxity with the compression group demonstrating these changes more quickly and with greater magnitude. Interestingly the discs demonstrated increased proteoglycan content in contrast to human disc degeneration, in which reduced proteoglycan content is observed during the degenerative process.

Kroeber et al. [51] developed a novel model that enabled the application of compressive force to the intervertebral discs of rabbits via attachment of an external loading device. Rabbit intervertebral discs were exposed to up to 28 days of loading of a disc compressive force equivalent to five times bodyweight. After 14 and 28 days of loading discs demonstrated significantly reduced disc space with annulus disorganization observed histologically. Increased dead cells were observed in the annulus and endplate. These changes were not reversible after an equivalent period of unloading.

Cyclical compression has also been investigated. In a rat tail model Ching et al. [45] investigated the effects of static and cyclical loading at 0.5, 1.5, or 2.5 Hz. Pins were inserted into the caudal 4th and 5th vertebrae. A compression device was applied to these pins. The greatest loss of interpin distance (a measure of intervertebral disc height and thus disc degeneration) was observed in rat tails subjected to static compression with the least loss of interpin distance, other than the sham control, observed in the 1.5 Hz group, suggesting disc response varies with the frequency of loading.

Instability. Various animal models of disc disease exist in which the intervertebral disc is exposed to increased instability at the motion segment promoting intervertebral disc degeneration. Approaches to produce instability include surgical resection of posterior elements such as facet joint and spinous processes [41] and the fusion of an adjacent level [52]. Miyamoto et al. [41] demonstrated that resection of spinous processes, supraspinous and interspinous ligament, with paravertebral muscle detachment accelerated cervical disc degeneration in a mouse model. At 12 months following surgical intervention the experimental group demonstrated advanced disc degeneration with intervertebral disc material herniation, AF disorganization, metaplasia of fibroblastic cells in the AF into chondrocytes, loss of disc height, and osteophyte formation.

Phillips et al. [52] reported a novel method of modeling intervertebral disc degeneration in the rabbit by performing simulated surgical spinal fusion at the lumbar L5–L7 level in rabbits. Spinal fusion eliminates movement at the index level but induces altered stresses at the adjacent mobile segments [91, 92]. The adjacent level intervertebral discs L4-L5 and L7-S1 demonstrated progressive disc degeneration. Annular disorganization with loss of normal collagen bundle arrangement was observed at three months. This was increased at six months and by nine months the normal structure of the disc had been replaced by disorganized fibrous tissue, annular tears, and loss of chondrocytes and notochordal cells in the NP were observed as was decreased monomeric size of the proteoglycans. Furthermore disc space narrowing, endplate sclerosis, and osteophyte formation were also observed in keeping with the clinical condition.

Instability studies allow an inducible method of progressive disc degeneration with many of the features observed in the clinical condition. The time course of progression of these models, requiring 9–12 months for the establishment of severe disc degeneration, may preclude their usage in studies of regenerative therapies given cost concerns.

3.3.3. Structural Models.
An alternate mechanism of inducing disc degeneration is directly compromising the structural integrity of the intervertebral disc. This task can be accomplished by chemical or direct physical methods.

Chemonucleolysis. Various chemical agents have been investigated as potential stimuli to induce the pathophysiological process of disc degeneration. The best described such agent, chymopapain, was first reported in clinical use in 1964 for the treatment of sciatica secondary to presumed disc protrusion [93]. Chymopapain is a proteolytic enzyme derived from the papaya latex [94] that produces disc degeneration by inducing proteolytic digestion or removal of glycosaminoglycan chains. Proteoglycan loss leads to disc height loss and altered biomechanical stability [26]. The enzyme selectively degrades intervertebral disc proteoglycan in a dose dependent fashion [59]. Inadequate doses may be followed by NP proteoglycan restoration [95]. High doses have also been demonstrated to directly produce annulus destruction in animal models [53].

Chondroitinase ABC is another enzyme demonstrated to produce disc degeneration in animal models [96]. Chondroitinase causes its effect by producing degradation of the polysaccharide side chains of the proteoglycans of the intervertebral disc [97]. Chondroitinase ABC injection was demonstrated to produce dose dependent intervertebral disc degeneration in a caprine model by Hoogendoorn et al. [98]. Chondroitinase ABC injection has also been used in an ovine model to induce intervertebral disc degeneration to enable assessment of regenerative therapies [65].

As described above injection of enzymes leads to proteoglycan loss, an essential component of the pathophysiological process of disc degeneration observed clinically. A criticism of the chondroitinase ABC for inducing disc degeneration is that the viability of native disc cells is largely preserved, enabling regeneration of the extracellular matrix [99].

Physical Methods. Surgical injury to the intervertebral disc is a well-established method of inducing disc degeneration. Injury can be performed to the endplate, the annulus, or the annulus and nucleus.

Endplate injury has been demonstrated in a porcine model to produce changes consistent with disc degeneration. Following lumbar endplate injury with a drill bit porcine intervertebral discs were observed over a 3-month period to demonstrate annular delamination, with reduction in nucleus proteoglycan content, cellularity and loss of gel-like structure [100]. Evidence of degeneration of varying degrees of magnitude was observed seven months following injury in a similar porcine model of endplate injury induced disc degeneration [101].

Annular injury models were first described in the 1930s by Keyes and Compere [55]. Keyes and Compere demonstrated that annular injury with subsequent NP expulsion leads to loss of disc height and degenerative changes at the index level. Following these pioneering studies multiple intervertebral disc injury methods have been investigated for their potential to induce disc degeneration. Broadly such methods can be considered under the categories of partial thickness annular injury and full thickness annular injury with nucleus involvement (see Figure 2). Full thickness annular injuries have the advantage of producing nuclear avulsion with relatively rapid degeneration. Partial thickness injuries produce a slower degenerative process.

Stab injuries and annulotomies have been performed in a variety of animal models including rats [46], rabbits [20], sheep [62], and pigs [61]. Osti et al. [62] demonstrated in an ovine model that partial thickness annular injury, consisting of a 5 mm depth incision that left the inner annulus and NP intact at the time of injury, would lead to progressive failure of the inner annulus with progressive disc degeneration over several months. Oehme et al. [63, 102] demonstrated in an ovine model that after three months a larger (20 mm × 6 mm) partial thickness annular injury resulted in significant increased disc height loss, increased MRI Pfirrmann degeneration scores, increased histological injury scores, and decreased NP glycosaminoglycans in the injured discs.
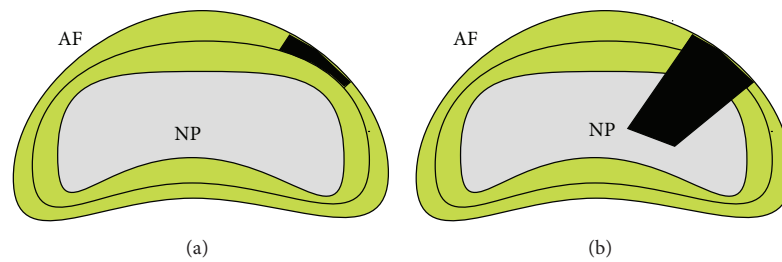
FIGURE 2: (a) Partial thickness annular injury. (b) Full thickness annular injury with NP involvement. AF indicates annulus fibrosus; NP indicates nucleus pulposus.
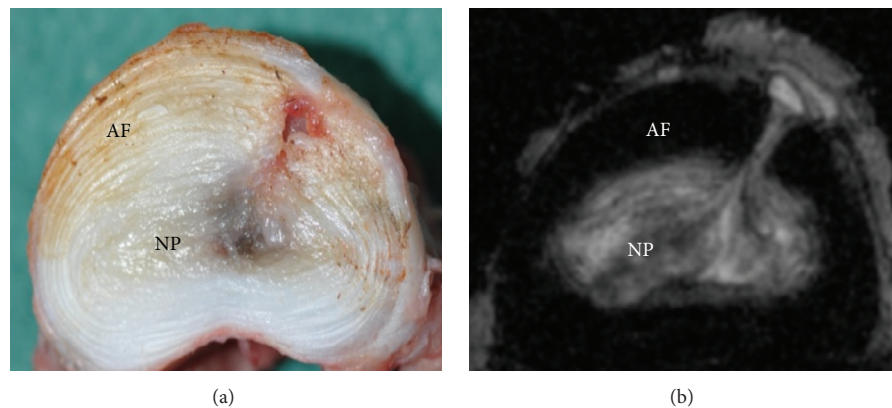

FIGURE 3: (a) Ovine drill bit injured intervertebral disc demonstrating injury penetrating through the annulus into the nucleus. (b) 9.4 T axial MRI T2 sequence demonstrating drill bit injury tract extending through AF to NP. AF indicates annulus fibrosus; NP indicates nucleus pulposus.

Full thickness intervertebral disc injury is demonstrated in the approach of Oehme et al. [23]. In this injury model a simulated partial lumbar microdiscectomy was performed by creating a 3×5 mm annular incision in ovine discs followed by removal of 0.2 g of intervertebral disc tissue, including NP. 24 weeks following performance of the partial-microdiscectomy injured and otherwise untreated intervertebral discs demonstrated increased disc height loss, increased MRI Pfirrmann degeneration scores, and reduced NP proteoglycan content relative to controls.

A novel full thickness intervertebral disc injury caprine model utilizing a drill bit has recently been described by Zhang et al. [22]. The authors compared scalpel blade annulotomy with insertion of a 4.5 mm drill bit to a depth of 15 mm. At two months the drill bit injured intervertebral discs demonstrated significantly increased histological injury scores relative to controls. This injury model has served as the stimulus for investigation in our laboratory utilizing an ovine model. The drill bit injury model has the advantage of producing a highly replicable injury demonstrated in the goat to produce disc degeneration over a two-month period. Drill bit injury was performed by insertion of a 3.5 mm drill bit 12 mm in depth in two adjacent ovine lumbar intervertebral discs. Sheep underwent necropsy at two months. Gross morphology and 9.4-tesla MRI demonstrated significantly increased injury scores in injured versus control discs (see Figure 3).

4. Involvement in Preclinical Trials

Despite the limitations of the animal models described above such models play an integral role in increasing our knowledge and understanding of the process of disc degeneration and in the development of novel therapies for clinical application. Given the complex pathophysiological process of disc degeneration with the interplay of cellular, biomechanical, and matrix components cellular therapy is considered by many to demonstrate the greatest potential in the treatment of this condition.

A recent review by Oehme et al. [103] comprehensively details the variety of preclinical and clinical trials of novel cell-based therapies for the treatment of lumbar intervertebral disc degeneration. Animal models used in preclinical trials of novel therapies include rat [47], rabbit [54], canine [56], porcine [61], ovine [23, 78, 79], and rhesus monkeys [104]. The vast majority of animal models described utilized full thickness annular injury with nuclear involvement to induce disc degeneration. As detailed above, the advantage of this injury model is the ability to consistently induce degeneration at a specified time point. Cell types investigated for regenerative potential include NP chondrocytes [56], bone marrow derived mesenchymal stem cells [54], and bone marrow derived mesenchymal precursor cells [23]. The three cell types detailed are notable for having demonstrated the ability

to promote intervertebral disc regeneration in preclinical trials with subsequent progression to clinical trials/series.

4.1. Clinical Translation. The EuroDISC clinical trial [105] investigated the transplantation of expanded autologous disc chondrocytes in patients undergoing single level discectomy. Interim analysis of 28 patients at 24 months revealed those patients who received chondrocyte transplantation reported greater pain reduction and demonstrated increased disc fluid content on MRI compared to controls. Percutaneous injection of expanded autologous mesenchymal stem cells in two small noncontrolled clinical trials leads to improved MRI T2 signal and clinical improvement [106, 107].

Autologous bone marrow mesenchymal stem cell administration has been investigated in two small series of patients [106, 107]. The trial of Orozco et al. [106] reported clinical improvement in 9 of 10 patients who received expanded autologous MSCs for treatment of low back pain with degenerative disc disease and failure of conservative treatment. The series of Yoshikawa et al. [107] consisted of two patients who at two years both reported significant improvement with improved disc hydration on MRI.

The administration of allogeneic mesenchymal precursor cells for the treatment of back pain has been investigated in a Phase II study [108]. A significantly greater proportion of MPC treated patients achieved minimal residual back pain and at least a 50% reduction in back pain. Phase III trials are now underway.

4.2. Pain. The discussion of clinical translation raises one of the most important considerations regarding the translation of findings from animal models of disc degeneration, that of pain. Disc degeneration causes the majority of its morbidity and disability through back pain—a subjective phenomenon. Pain is a symptom experienced by patients and is multifactorial in nature. The clinical observation of significant radiological disc degeneration in the absence of significant back pain in many patients is suggestive of the notion that the two are not necessarily well correlated at all times. Thus the measures of disc degeneration employed in animal models of disc disease such as histology and radiological degeneration scores and macroscopic and biochemical analysis can serve as useful markers of underlying disc degeneration but can inform the observer to only a limited degree of the likely disability associated with such findings.

The assessment of intervertebral disc degeneration related pain in animal models is still in its relative infancy. The majority of such research has been conducted in rodent models [109, 110]. Pain in rodents can be assessed in three ways [110]: observation of pain-related behaviours (e.g., increased grooming and "wet-dog shakes" [111]), measuring functional performance (e.g., locomotor ability assessment in mice [109]), or determining response to mechanical or thermal stimuli (e.g., grip strength in response to axial stretch, a possible measure of axial low back pain). A recent study comparing sensitivities of different pain assessment methods in a rat model suggested hind paw mechanical sensitivity and duration of grooming as the most sensitive measures of degeneration induced pain [110]. Hind paw mechanical sensitivity

offers the advantage of enabling analysis of threshold changes whereas spontaneous behavioural change may better relate to the presence of pain and general condition of an animal [111, 112]. Performance on functional assessments, such as the rotarod test, also declines following lumbar intervertebral disc injury [46].

The assessment of pain in small animal models is imperfect but greatly increases our power to investigate the underlying pathophysiology of intervertebral disc degeneration related pain.

5. Conclusions

The complexity of the human intervertebral disc bares repetition. Given this inherent complexity no animal model will perfectly replicate the clinical condition. The best that can be hoped for is to mimic as closely as possible the clinical condition of degenerative disc disease. Important considerations in choosing an appropriate animal model are the absence of notochordal cells, animal and intervertebral disc size relative to humans, biomechanical forces acting upon the intervertebral disc, mechanistic concerns (i.e., mechanical injury versus chemical injury), and ethical considerations. Nonhuman primates closely match the clinical condition with regard to many of the physical and mechanistic criteria, particularly given the demonstration of spontaneous intervertebral disc degeneration in baboons and macaques. However, ethical considerations should preclude their widespread utilization.

The ovine model of disc disease possesses many desirable characteristics when considering the ideal intervertebral discs model: absence of notochordal cells, similar body mass to humans, and similar biomechanical forces acting upon the intervertebral disc. A major potential criticism of this model is the quadrupedal rather than bipedal nature of sheep. As discussed previously biomechanical studies have indicated that the ovine and human lumbar spines have good comparability in many biomechanical properties [113] in spite of the quadrupedal/bipedal dichotomy.

Certain questions will remain unanswerable in large animal models without significant advances in technology. As such, a role for small animal models will continue, particularly in the investigation of the action of specific gene products in disc degeneration through the use of genetically modified/knockout mice.

The variety of methods of inducing disc degeneration is even broader than the variation in animal models. Spontaneous models of disc degeneration, such as the chondrodystrophic dog and primate, are most likely to parallel the clinical condition in terms of underlying mechanism and time frame. However, the variability of onset and prolonged time course of the degenerative process renders such models difficult to utilize in many contexts. Investigation of regenerative therapies, for example, would be rendered exceptionally difficult if an investigator were to wait for all animals scheduled for investigation to spontaneously develop an appropriate degree of degeneration. For investigations of regenerative therapies it is thus likely that methods of inducing structural injury will be the most utilized as these enable instantaneous production of a replicable injury at a defined time point.

In conclusion no animal model will mimic the clinical condition of disc degeneration with complete fidelity. This is due to the complexity of clinical intervertebral disc degeneration and the immense influence of the subjective phenomena of pain in determining patient outcomes. Animal models will continue to play an essential role in refining our understanding of the pathophysiology of disc degeneration, developing novel therapies for this condition, and ultimately translating such therapies to the clinic.

Competing Interests

The authors have no competing interests to disclose with regard to this paper.

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Chapter 2.   Materials and Methods

This chapter presents the structural outline of the thesis as well as detailed descriptions of the material and method used to perform and analyse the experiments described in the following chapters. Materials and methods specific to individual experiments are discussed in each experimental chapter.

Included in this chapter is a manuscript published in the Journal of Visualized Experiments (JoVE), **"Ovine Lumbar Intervertebral Disc Degeneration Model Utilizing a Lateral Retroperitoneal Drill Bit Injury,"** describing the pre-operative care, anaesthetic and post-operative care and surgical technique to approach the lumbar intervertebral disc and perform the drill bit injury. The candidate, Chris Daly, contributed to the experimental design, writing of the manuscript, revision of the manuscript and starred in the accompanying video. Proportional contribution of co-authors are explained in the signed declarations on page xvi.

The description of materials and methods is divided into pre-clinical and clinical components in keeping with the structure of this thesis.

2.1. Purpose

The purpose of this thesis is to re-engineer lumbar discectomy surgery through a combination of pre-clinical and clinical initiatives. As the translation of preclinical research to clinical application was always the intention, the methodology of the preclinical work employed closely relates to contemporary surgical practice. The inclusion of clinical studies provides for an immediate application and positive impact on patients undergoing lumbar discectomy surgery.

2.2. Study Design

The experimental design of this thesis is thus divided into preclinical and clinical components. The initial preclinical investigation was performed in order to identify and characterize a suitable large animal model of the post discectomy lumbar intervertebral disc. Expanding on this initial study, the second preclinical investigation utilizes the same annulotomy discectomy injury model in order to assess the safety and efficacy of pentosan polysulfate (PPS) primed allogeneic mesenchymal progenitor cells (pMPC) to regenerative lumbar intervertebral discs following lumbar discectomy.

The first clinical component of the thesis consists of a survey used to establish the prevailing lumbar discectomy peri-operative practices amongst Australasian spine surgeons. The final clinical experimental chapter describes the protocol of a randomised clinical trial to assess the role and impact of post-operative activity restrictions in contemporary clinical practice. More detailed experimental protocols are outlined in the respective chapters.

2.3. Mesenchymal Progenitor Cells (MPC)

2.3.1. MPC Manufacturing Process

The ovine STRO-3$^+$ Mesenchymal Progenitor Cells used in the experiments in Chapter 4 were generously provided by the laboratory of Professor Andrew Zannettino from the University of Adelaide. A stock batch of the ovine MPCs used for these studies were prepared from iliac crest bone marrow aspirates of adult Border Leicester Merino crossbred sheep, screened for mycoplasma and other ovine pathogens using our published procedures (1). In brief, the mononuclear cell fraction cells were separated from other cells using density gradient centrifugation (2). The mononuclear cell fractions were transported on ice to the MPC isolation facility. MPCs were isolated using immunoselection with monoclonal antibodies to STRO-3$^+$ in combination with magnetic cell sorting using IgG conjugated magnetic Dynabeads(3). The MPCs were then expanded to passage five (P5). The lineage authenticity of these MPCs was confirmed using multilineage differentiation assays and flow cytometry, demonstrating that the MPCs expressed the characteristic MSC markers CD73, CD90, CD105, CD44 and CD 146, but exhibited low expression of the haemopoietic and vascular endothelial markers: CD14, CD34 and CD45. Following culture, total cell count and cell viability were determined and

MPCs were suspended in cryopreservative medium (42.5% ProFreeze/7.5% DMSO/50% Alpha MEM) to the required concentration, cryopreserved, and stored in the vapour phase of a long-term liquid nitrogen until the time of use.

2.3.2. Priming of MPCs

Cells were thawed and viability confirmed as described in section 2.3.4. 1 x10^6 MPCs were seeded into 24 well plates and incubated with high glucose DMEM (Dulbecco's Modified Eagle Medium) containing 5µg/ml PPS for 24 hours. Following 24 hours of culture, media was removed from all wells and cells were washed twice with Phosphate Buffered Saline (PBS). Total cell count and viability were again confirmed as described below. The PPS primed MPCs (pMPC) were then suspended in cryopreservative medium (42.5% ProFreeze/7.5% DMSO/50% Alpha MEM) to the required concentration, cryopreserved, and stored in the vapour phase of a long-term liquid nitrogen until the time of use. This protocol is described in more detail in Wu et al.(4) in Appendix 1.

2.3.3. Preparation of MPCs Prior to Administration

Frozen MPCs and pMPCs were transferred to the surgical facility (Monash Animal Services) and vials were rapidly thawed in a 37ºC water bath immediately prior to administration. Vials were checked for damage or leakage. Cell preparations were inspected for cell clumping, discolouration or cloudiness and, if present, the cells were discarded.

Cell viability and number were determined using a Neubauer haemocytometer in the following manner. After thawing the vials, 10 µL of MPC solution was diluted in 10 µL of 0.4% (v/v) trypan blue solution. A glass cover was placed over the haemacytometer chamber. 10 µL of the MPC/trypan blue solution was pipetted into each side of the haemacytometer chamber. Using a microscope with a 10x ocular lens, a live/dead cell count was performed. The number of cells per mL and the total number of cells in the original culture was calculated. If cell counts did not fall within +/-25% of that specified on the ampoule label, the vial was discarded. Percentage viability was calculated. If the cell viability was less than 85% a replacement ampoule was used.

2.4. Cell Carriers and Cell Delivery Vehicles

The cell carriers and delivery vehicles utilized in the preclinical trial described in Chapter 4 were selected due to their pre-existing clinical approval and experimental evidence demonstrating the ability to promote intervertebral disc regeneration(6). Pre-existing clinical approval was deemed essential to facilitate clinical translation.

Pentosan Polysulfate (PPS): Supplied by *Bene Pharmachem Gmb H & Co, Geretscried, Germany.*

Gelfoam® absorbable gelatin sponge: Supplied by *Pharmacia & Upjohn Co*, division of *Pfizer Inc, New York, USA*. Stored at sterile at room temperature until use.

Tisseel® fibrin glue: Supplied by *Baxter Ltd Australia*. Produced by *Baxter Healthcare Corporation Westlake Village, CA, USA*. Stored frozen at -20°C until thawed for immediate use.

2.5.   Experimental Animals

2.5.1.   Animal Type

Species: Ovis Aries (sheep)

Strain: Border-Leicester Merino crossbred sheep

Source: Monash Animal Research Platform, Monash University, 246 Clayton Rd, Clayton, Vic, AUS

Gender: Female

Age: 2 – 4 years of age

Weight: The weight range of the sheep was from 55-75kg.

Identification: The sheep were ear tagged for identification, and tag identification numbers were recorded on all study related documentation.

2.5.2.   Animal Husbandry, Housing and Diet

All animal experiments were conducted at Monash Animal Services, Monash University Clayton, Victoria, Australia. At all times sheep were under the supervision of suitably experienced and qualified animal house staff, including a qualified veterinary surgeon. All sheep were open pasture raised and freely ambulating prior to the trial. Animals underwent comprehensive veterinary review prior to inclusion in the study and acclimatised at Monash Animal Services for a period of two weeks prior to surgical intervention. After surgical intervention, animals were held for observation for up to one week in deep litter pens. Water was available *ad libitum* and sheep were fed Lucerne hay twice weekly with grass hay provided on all other days. Once the animals were deemed sufficiently recovered from surgery they were transferred to Monash Animal Services Churchill Gippsland Campus and placed in pastures.

2.6.   Surgical Procedure

The following manuscript published in the Journal of Visualized Experiments (JoVE), **"Ovine Lumbar Intervertebral Disc Degeneration Model Utilizing a Lateral Retroperitoneal Drill Bit Injury"** describes the pre-operative care, anaesthetic protocol, post-operative care and surgical technique

used to approach the lumbar intervertebral disc and perform the drill bit injury. The same surgical approach to the intervertebral disc was utilised to perform the annulotomy injury model. The surgical technique used to perform the annulotomy injury is described following the JoVE manuscript.

Video Article

Ovine Lumbar Intervertebral Disc Degeneration Model Utilizing a Lateral Retroperitoneal Drill Bit Injury

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Abstract

Intervertebral disc degeneration is a significant contributor to the development of back pain and the leading cause of disability worldwide. Numerous animal models of intervertebral disc degeneration have been developed. The ideal animal model should closely mimic the human intervertebral disc with regard to morphology, biomechanical properties and the absence of notochordal cells. The sheep lumbar intervertebral disc model fulfils these criteria. We present an ovine model of intervertebral disc degeneration utilizing a drill bit injury through a lateral retroperitoneal approach. The lateral approach significantly reduces the incision and potential morbidity associated with the traditional anterior approach to the ovine spine. Utilization of a drill-bit method of injury affords the ability to produce a consistent and reproducible injury, of precise dimensions, that initiates a consistent degree of intervertebral disc degeneration. The focal nature of the annular and nucleus pulposus defect more closely mimics the clinical condition of focal intervertebral disc herniation. Sheep recover rapidly following this procedure and are typically mobile and eating within the hour. Intervertebral disc degeneration ensues and sheep undergo necropsy and subsequent analysis at periods from eight weeks. We believe that the drill bit injury model of intervertebral disc degeneration offers advantages over more conventional annular injury models.

Video Link

The video component of this article can be found at https://www.jove.com/video/55753/

Introduction

Lower back pain is the leading cause of disability worldwide[1]. Lumbar intervertebral disc degeneration associated discogenic pain is considered a significant contributor to lower back pain[2]. There is an increasing demand for reliable animal models of intervertebral disc disease for broadening the understanding of the degenerative process and for the investigation of potential therapies.

Numerous animal models of intervertebral disc degeneration exist[3]. Animals models used in the investigation of degenerative disc disease range in size from mice[4], to larger mammals such as dogs[5], sheep[6], and non-human primates[7]. Methods used to induce intervertebral disc degeneration can be broadly classified into the categories of mechanical (*e.g.* intervertebral disc compression[8] or surgical injury[6]), chemical (*e.g.* chemical nucleolysis[5]) or, less commonly, spontaneous degeneration (*e.g.* the sand rat[9]).

Given the complexity of human intervertebral disc degeneration, a perfect animal model does not exist. However, important considerations in choosing an appropriate animal model to mimic this condition closely have been identified[3]. Such considerations include the absence of notochordal cells (primitive cells with possible progenitor cell function[10] absent from the adult nucleus pulposus in humans, sheep, goats and chondrodystrophic dogs but present in most mammals), similarities in animal and intervertebral disc size relative to humans, comparable biomechanical forces to the clinical condition, mechanistic and ethical considerations[3].

Non-human primates meet many of the above criteria. Baboon and macaque models of spontaneous intervertebral disc degeneration have been described[11,12,13]. Both species spend large amounts of time in erect or semi-erect postures — a distinct advantage relative to other animal

models. However, ethical and practical consideration (*e.g.* expense, housing, delayed onset of spontaneous degeneration) restrict their use in many institutions.

The ovine spine is an established model of intervertebral disc degeneration, with advantages including cellular, biomechanical and anatomical similarities to the human spine[10,14,15]. Despite the quadrupedal stature of sheep the ovine lumbar intervertebral disc is exposed to similar stresses to the human disc[14]. The ovine model is also more widely accepted, from an ethical perspective, than non-human primate models. Varied methods have been described to initiate the degenerative process, many of which require direct access to the intervertebral disc. Due to the termination of the spinal cord in the sacral region and ossification of the posterior longitudinal ligament in the ovine lumbar spine, posterior approaches to the intervertebral disc are technically challenging and less commonly used in the sheep[16]. The traditional access routes to the sheep lumbar spine, *i.e.* via anterior or anterolateral approaches, require large abdominal incisions, are fraught with risks of hernia, and damage to internal viscera and neurovascular structures[16]. The use of a relatively small lateral incision away from dependent abdominal areas may decrease such risks[17].

We present an ovine model of degenerative lumbar intervertebral disc disease using drill bit injury performed through a minimally invasive lateral approach, and inspired by the work of Zhang *et. al*[18]. The goal of this protocol is to enable a reliable lumbar disc injury model that is readily reproducible, produces a consistent injury, and is safe and well tolerated. This approach is well-suited to investigators seeking to induce a milder degree of lumbar intervertebral disc degeneration than that observed with traditional surgical annulotomy (unpublished data) for the investigation of either intervertebral disc degeneration or regenerative therapies. These findings will be described in a forthcoming publication.

Protocol

The protocol detailed in this manuscript follows the animal care guidelines of Monash University Animal Ethics. Animal ethics approval for this protocol has been granted by Monash University Animal Ethics. Ethics approval number: MMCA/2014/55

1. Sheep Preparation

NOTE: Ewes aged two to four years were used.

1. Fast sheep for 18 h prior to anesthesia. Provide animals with access to water until 6-12 h prior to operation[19].
2. Sedate animals by intravenous injection of medetomidine hydrochloride (0.015-0.020 mg/kg) to facilitate transfer to operating suite.
   NOTE: The medetomidine hydrochloride serves to reduce animal stress and agitation associated with separation from other animals for transfer to the operating suite.
3. Inject thiopentone (10-13 mL/kg) for induction of anesthesia upon arrival to the operating suite.
4. Administer prophylactic intravenous antibiotics (amoxicillin 1 g IV) immediately following thiopentone injection.
5. Intubate sheep using a size 7.5-9 mm (internal diameter) endotracheal tube[20].
6. Maintain anesthesia using inhaled isoflurane (2-3%) in 100% oxygen at a flow rate of 2 L/min. Attach a pulse oximeter to the sheep's ear.
7. Closely monitor the sheep's vital signs (heart rate, respiratory rate and oxygen saturation via pulse oximeter and observation) and level of consciousness.
   NOTE: Indicators of light anesthesia such as spontaneous chewing, active regurgitation, and spontaneous movements should prompt increase in the level of anesthesia. Red flag signs indicating urgent lightening of anesthesia include respiratory compromise and severe bradycardia. Rotation of the eye is not a consistent indicator of depth of anesthesia in sheep[19].

2. Disc Level and Incision

1. Collect the surgical tools needed for this procedure: veterinary clippers, 20 mL luer-lock syringe, 21G IV Needle, #4 scalpel handle, #22 scalpel blades, Gillies tissue forceps, Metzenbaum curved dissecting scissors, Deaver retractor, Hohmann retractor blade, 3.5 mm Brad point drill bit, drill bit stop, drill, autoclavable veterinary drill bag, needle holder, 2-0 absorbable synthetic braided sutures, 3-0 absorbable synthetic braided suture and Mayo suture scissors.
2. Prepare the operating suite. Clean the operative table and instrument stand with 70% ethanol. Autoclave all surgical instruments prior to operation. Perform pre-operative anesthetic check.
3. Place the sheep on the operating table in the right lateral position.
4. Using electronic clippers, shave the region defined superiorly by the lower ribs, inferiorly by the iliac bone, medially by the contralateral lumbar transverse processes and approximately 10 cm lateral to the ipsilateral lumbar transverse processes.
5. Palpate the iliac crest, lumbar transverse processes (L1-6) and costo-vertebral angle for the landmarks for surgical incision site. Mark these landmarks with a sterile pen.
6. Prepare the lateral abdomen by disinfecting with chlorhexidine and alcoholic-iodide antiseptic wash.
7. **Observe standard surgical aseptic techniques throughout the operation. The surgical team scrubs prior to the operation. Place a sterile fenestrated square drape over the surgical site, and a large sterile square drape on the overhead table.**
   1. Sterilize all items to be used within the operative site prior to the operation. Monitor and maintain sterility of the surgical site throughout the operation. Ensure all items introduced into the sterile field are sterile and transferred in a sterile fashion.
8. Use surgical loupe magnification and a headlight to facilitate visualization during the surgical procedure.
9. Make a longitudinal incision using the #22 scalpel blade attached to the #4 scalpel handle parallel to and 1 cm anterior to one to two lumbar transverse processes above and below the intervertebral disc levels of interest.
   NOTE: Further information regarding incision planning can be found in the discussion.
10. Use the monopolar diathermy to divide the underlying subcutaneous tissue and the lateral aspect of the abdominal wall musculature; direct the dissection towards the tips of the lumbar transverse processes above and below the intervertebral discs of interest.
11. Divide the thoracolumbar fascia longitudinally at its attachment to the transverse processes.

12. Visualize and preserve the underlying *quadratus lumborum*, psoas muscles and the traversing neurovascular bundles.
13. Maintain hemostasis through the procedure using diathermy.
14. Sweep the fingers between the plane of the peritoneum and posterior abdominal wall musculature at the exposed intervertebral disc levels to perform digital blunt dissection.
15. Retract the *quadratus lumborum* and psoas muscles posterolaterally using a Deaver retractor to further expose of the intervertebral discs.
16. Palpate for the concave intervertebral bodies and the convex intervertebral discs.
17. Position the retractors immediately over the discs and take care to ensure lumbar vessels are not damaged.
18. Using surgical loupe magnification with a headlight, identify the lumbar vessels which are located approximately 1 cm caudal to the inferior endplate.
19. Perform an intraoperative lateral X-ray to confirm the disc level.[21]
    Note: Radiograph settings: 47kV; 4 mAs[21]
20. **Depending on the disc levels desired, expose the intervertebral disc by separating the surrounding structures and attachments as below.**
    1. For levels L3/4 and above, sweep aside the muscular attachments over the disc using digital blunt dissections.
    2. For levels L4/5 and below, sharply divide the thicker tendinous muscular attachments over the disc using bipolar diathermy and scissors.
       NOTE: L6/S1 disc can be difficult to access due to obstruction by the iliac crest. If access cannot be accomplished via the lateral approach an anterior approach may be utilized.

3. Drill Bit Injury

NOTE: Pre-operative planning includes the allocation of injury/treatment levels and control levels. Further information regarding level allocation can be found in the discussion.

1. Define the drill bit entry point by observing the left lateral and anterior extremities of the intervertebral disc.
   NOTE: The entry point is located at the midpoint of this left anterolateral quadrant (defined by the anterior and lateral extremities of the disc). The drill bit is inserted at this entry point with a trajectory aimed towards the center of the intervertebral discs and directed slightly cranial to perpendicular.
2. **Fit a Brad-point drill bit into the power drill. Ensure the diameter of the drill bit is slightly less than the intervertebral disc height *i.e.* ~3.5 mm for lumbar intervertebral disc in 60-70 kg sheep.**
   1. Apply a drill bit stop to provide an unprotected drill bit length of approximately half the diameter of the lumbar intervertebral disc *i.e.* ~12 mm for lumbar intervertebral discs in 60-70 kg sheep.
3. Apply the drill bit to the entry point and direct it in a trajectory slightly cranial to the center of the intervertebral disc. The slight cranial angulation is to minimize the risk of endplate injury.
4. Advance the drill bit slowly into the intervertebral disc with the drill on low power for 1 s. Adjust the trajectory in a slight cranial or caudal fashion if excessive resistance is encountered
   NOTE: Such excessive resistance likely indicates contact with the endplate.

4. Closure

1. Once hemostasis is achieved, irrigate the wound with Ringers' solution.
2. Perform layered closure, preferably using 2-0 absorbable synthetic braided sutures to the lateral abdominal wall tissues and continuous 3-0 undyed absorbable synthetic braided subcuticular suture to the skin.

5. Post-operative Management

1. Place a fentanyl transdermal patch (75 µg/h) in the inguinal region for post-surgical analgesia for 3 days.
2. Additionally, use intravenous buprenorphine (0.005-0.01 mg/kg) for top-up analgesia if needed.
3. Cease the inhalational anesthetic. When spontaneous breathing occurs, remove the endotracheal tube.
4. Allow the animal to recover in a holding cage under constant observation.
   NOTE: The animal should not be left unattended until it has regained sufficient consciousness to retain sternal recumbency.
5. Once the animal is fully alert and standing, re-introduce food and water. Once fully recovered, return the animal to its operative facility holding pen with other animals.
6. Monitor closely for 24 h and continue observation for one week. Monitor for evidence of post-surgical pain or distress.
   NOTE: Post-operative transdermal fentanyl patch applied for three days should provide sufficient analgesia. Additional analgesic requirements should prompt animal review.
7. Feed the sheep normally, and allow the sheep to carry out normal activities without restriction. Observe the sheep for any evidence of neurological deficit such as lameness.
   NOTE: The intervertebral disc defect produced by the drill bit injury method is on the anterolateral aspect of the disc and the injury depth is limited by the drill bit stop to the mid nucleus. As the neural elements are located posterior/posterolateral to the intervertebral disc, the risk of neural compromise secondary to symptomatic nucleus pulposus is remote. This anatomical characteristic of the model precludes the use of neurological examination to distinguish intervertebral disc degeneration with and without nucleus pulposus herniation.
8. Return the sheep to the university farm to await euthanasia and necropsy at the end of the experimental period.

6. Euthanasia

1. Perform sheep euthanasia at an appropriate time interval following drill bit intervertebral disc injury.
2. Inject intravenous pentobarbitone sodium (>100 mg/kg) for euthanasia.

Representative Results

Pre-operatively, sheep underwent baseline 3T magnetic resonance imaging (MRI) for assessment of underlying intervertebral disc morphology and degeneration. Sheep underwent additional intra-operative lateral radiography for confirmation of intervertebral disc level and calculation of disc height index. A pre-operative sagittal plane slice from 3T MRI and an intra-operative radiograph are demonstrated in **Figure 1**.
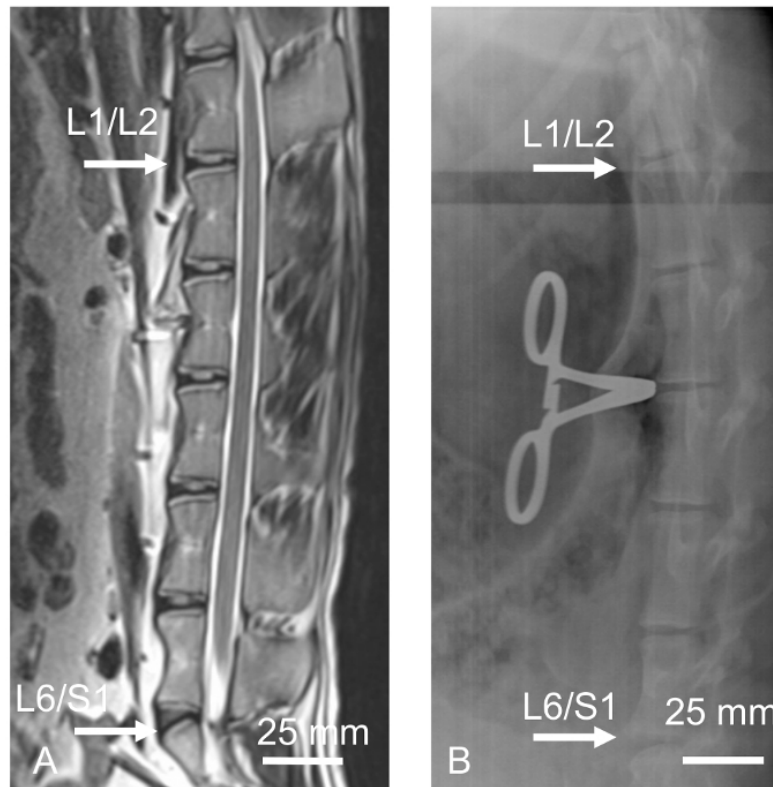

Figure 1: Pre-operative 3T MRI (**A**) and Intra-operative Lateral Radiograph (**B**). (**A**) Sagittal slice from 3T MRI (3T T2-weighted spin echo sequence) of ovine lumbar spine demonstrating lumbar 1/2 (L1/2) to lumbosacral (L6/S1) intervertebral discs. Intervertebral discs have a homogenous hyperintense appearance indicating no evidence of significant pre-operative intervertebral disc degeneration. Note that the ovine lumbar spine normally has six lumbar vertebrae, and the ovine spinal cord terminates in the sacral region. (**B**) Intra-operative lateral radiograph (settings: 47 kV; 4 mAs) demonstrating L1/L2 and L6/S1 intervertebral discs with the surgical instrument marking the L3/L4 intervertebral disc. Scale bars = 25 mm. Please click here to view a larger version of this figure.

Following the surgery, sheep typically recovered and were independently mobile within 1 h. Sheep were observed closely for one week, and subsequently returned to farmland until necropsy at 8 weeks following intervertebral disc injury. No adverse events occurred. At 8 weeks following disc injury, sheep underwent necropsy, X-ray and MRI of lumbar spines, and processing of discs for histological and biochemical analysis.

Representative post-operative images of the gross morphological appearance, and radiological 9.4T MRI images of injured sheep lumbar intervertebral discs at 8 weeks (56 days) post injury are shown in **Figure 2**. The gross morphological image demonstrates the drill bit injury tract penetrating the annulus fibrosus and extending into the nucleus pulposus. This is also evident in the 9.4T MRI. Comprehensive description and analysis of the outcome of this approach will be described in a forthcoming publication detailing the model validation study.
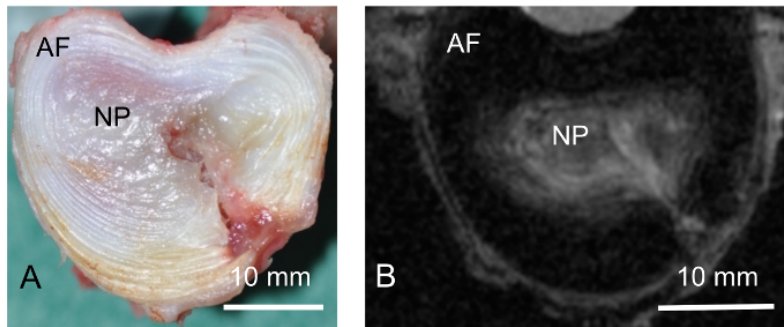
Figure 2: Gross Morphological and MRI Images of Injured Disc. (**A**). Gross morphological image of intervertebral disc demonstrating injury tract penetrating annulus fibrosus (AF) and extending into nucleus pulposus (NP). (**B**). 9.4T MRI (T2-weighted fast spin echo sequence) also demonstrating injury tract penetrating through AF into NP. Scale bar = 10 mm. Please click here to view a larger version of this figure.

Discussion

This minimally invasive lateral access approach is efficacious and safe with no post-operative herniae, abdominal wound dehiscence or infection observed in this series. Use of the drill bit intervertebral disc injury model with a depth stop provides a reproducible method of inducing a consistent intervertebral disc injury of known dimension (*i.e.* a 3.5 mm diameter x 12 mm depth injury in this study). In our experience, this method produces a less severe degree of disc degeneration than that observed in conventionally described ovine scalpel blade lumbar intervertebral disc annulotomy models[6,22] (unpublished data). This will be described in a forthcoming publication.

In making the initial longitudinal skin incision (step 2.9), the exact length and location of the incision should be modified based on the desired disc levels. More superior disc levels (T12/L1) can be reached by extending the incision to the costovertebral angle, whilst an incision extending to the iliac crest will allow access to the lower lumbar spine (to L5/L6). A 10 cm cut will facilitate access to three to four disc levels, while a smaller focused incision at 5 cm is necessary for access to single-discs. We prefer to perform injury at two levels, usually L2/L3 and L3/4. This enables the adjacent L1/2 and L4/5 intervertebral disc levels to be utilized as non-injured internal controls. Once technically confident, the surgical procedure on one sheep can be completed in less than one hour with minimal blood loss and discomfort[18]. The critical step and major technical challenge of this technique is the avoidance of endplate injury during drill bit disc injury. Clearly defining the superior and inferior margins of the intervertebral disc at the entry point of the drill-bit, is of the utmost importance. Slowly progressing the drill on low speed into the intervertebral disc, starting approximately perpendicular with slight cranial angulation also minimizes the risk of endplate injury. Lengthening of the skin incision may be required to obtain sufficient angulation of the drill.

Simple modifications to this technique include changes in drill bit size and depth, as these will be dictated by the size of the animal and lumbar intervertebral discs. This approach can be used to reliably induce degeneration in the intervertebral discs from T12/L1 to L5/6. The retroperitoneal approach may be used to access the intervertebral disc to induce degeneration by other mechanisms[16] or administer experimental therapeutic agents.

Limitations of this approach relate to the extent of the intervertebral disc injury and subsequent degeneration induced by this approach. If an investigator seeks to induce severe intervertebral disc degeneration, other more aggressive methods of disc injury such as scalpel blade annulotomy[6] should be considered. The acute defect produced in the intervertebral disc by the drill bit method of injury is relatively small, and may not be well suited to the administration of therapeutics at the time of injury.

The ovine spine was chosen for the intervertebral disc injury model for several reasons. Non-human primates, despite their anatomical and biomechanical similarities to the clinical condition (*i.e.* large amounts of time in erect and semi-erect postures), present sufficient ethical and practical considerations to prevent their utilization in many institutions. Although a quadruped, the sheep lumbar intervertebral disc is anatomically comparable and exposed to similar biomechanical stresses to human lumbar intervertebral disc[16,18]. Sheep demonstrate the loss of notochordal cells from the nucleus pulposus in early adulthood, as do humans[10,23]. Notochordal cells may have progenitor cell function and have been demonstrated to influence the course of disc degeneration through regeneration of the disc matrix. Finally, from a pragmatic perspective, sheep are hardy animals able to tolerate surgery well, are readily available, and present an economically feasible option[16,18].

The goat[18] is another animal model of lumbar disc degeneration that presents many of the advantages of the sheep model – similar size, economic feasibility, resilience, and absence of notochordal cells in the adult[24]. Other large animal models present additional challenges - the presence of notochordal cells in the porcine model, and ethical issues that may be associated with canine models. For a comprehensive review of animal models of intervertebral disc degeneration, the reader is directed to a recent review by Daly *et. al*[3].

As the ovine intervertebral disc demonstrates spontaneous loss of notochordal cells and undergo progressive degeneration with age[23], it is imperative to ensure consistency of sheep age in experiments. We prefer to use ewes aged two to four years, as at this age, notochordal cells are now absent[23]. From our own experience, minimal spontaneous degeneration has occurred in sheep aged from two to four years despite the loss of notochordal cells. Furthermore, the sheep vertebral body growth plate closes at approximately 24 months with vertebral body growth having ceased months earlier[25], minimizing the risk of any influence on disc regeneration from adjacent growth plate cells. Ewes were preferred because they are less aggressive than their male counterparts facilitating easier animal handling. If male sheep are used, we recommend using wethers.

In a study by Zhang[18] using a similar method of drill bit injury, where a drill bit measuring 4.5 mm in diameter was inserted 15 mm deep with manual rotation of 360° to produce disc degeneration in goats, there was no statistically significant difference in radiographic Pfirrmann degenerative score in the injured discs as compared to preoperative images. There was, however, demonstrable histological evidence of mild to moderate disc degeneration[26]. In contrast in this study, gross morphological and 9.4T MRI analysis revealed evidence of significant degenerative changes in the lumbar intervertebral discs, indicating the significant advantage of this approach.

The application and outcome of this method will be described in a forthcoming publication comparing the drill bit method of intervertebral disc injury to the established annulotomy method in the ovine model. This method may also be used in future for the investigation of regenerative therapies.

Disclosures

The authors declare they have no competing financial interests to disclose.

Acknowledgements

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2.6.1. Annulotomy Injury

The microdiscectomy annulotomy injury was performed following surgical exposure of the intervertebral disc, as described in the manuscript **"Ovine Lumbar Intervertebral Disc Degeneration Model Utilizing a Lateral Retroperitoneal Drill Bit Injury,"** and as described by Oehme et al. (5,6). Following surgical exposure of the lumbar intervertebral disc of interest via a lateral retroperitoneal approach (see Figure 1), the annulotomy injury was performed by creating a 3mm x 5mm window incision in the annulus using a pituitary rongeur. The disc tissues collected (200.0 +/- 3.0 mg) consisted mainly of annulus fibrosus (AF) and the outer region of the nucleus pulposus (NP). The adjacent L1-2 and L4-5 discs served as untreated controls. Animal recovery is described in the included manuscript above.
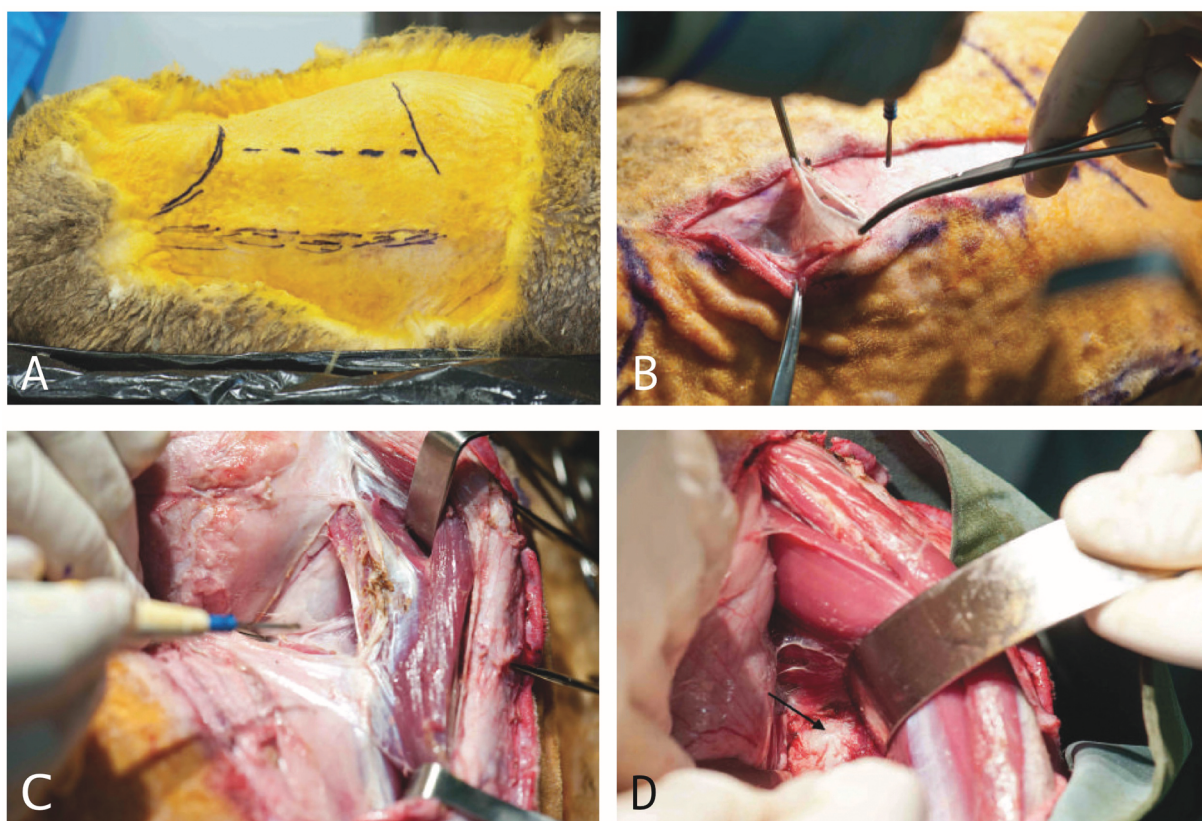
Figure 1. A. Preoperative photo of the sheep in the right lateral position with the iliac crest (left curve lined), costal margin (right straight line), tips of the transverse process (upper dashed line) and spinous processes (lower dashed line) marked. **B. Longitudinal skin incision** made anterior to transverse process tips. **C. Division of the thoracolumbar fascia** at the tips of the transverse processes. **D. Intervertebral disc revealed (arrow)** following retraction of the psoas muscle laterally abdomen retracted medially. Diagram is taken from Oehme et al. (5).

2.7. Post Mortem Analysis

At six months following surgery, all animals were euthanased by intravenous injection of 150 mg/kg of pentobarbital (Sigma-Aldrich, Castle Hill, NSW, Australia) under veterinarian supervision. The lumbar

spines were then removed *en bloc*, a segment was isolated from the mid-sacrum to the thoracolumbar junction, placed on ice and transferred to Monash Biomedical Imaging for MRI analysis (Siemens Skyra Widebore 3T MRI, Siemens, Erlangen, Germany and Agilent 9.4T MRI Small Animal Scanner Agilent/Varian, Santa Clara, CA, USA). All spines were subjected to radiological analysis using conventional x-ray radiography and MRI. Spinal columns were then transected in the horizontal plane through their vertebral bodies using a band saw to yield spinal segments that included complete lumbar discs with the adjacent half of vertebral bodies attached. Subsequent gross morphological, biochemical and histological analysis of discs were undertaken using these spinal segments as described below. Spinal segments containing discs destined for histological analysis were transferred to phosphate buffered formalin.

2.8. Radiographic Analysis

2.8.1. X-ray Radiographic Analysis

Lateral lumbar spinal digital x-rays (Radlink, Atomscope HF200A, Redondo Beach, CA, USA) of all sheep were obtained intra-operatively, prior to disc injury, and at the time of post-mortem assessment with the lumbar spine in situ.

2.8.2. MRI Analysis

All animals underwent pre-operative 3T MRI under general anaesthetic (Siemens Skyra Widebore 3T MRI, Siemens, Erlangen, Germany) and post-operative 3T and 9.4T MRI at the time of general anaesthetic at Monash Biomedical Imaging (MBI, Melbourne Australia). Due to the high-quality images available with the 3T MRI and the additional benefit of elimination of parallax error disc height index was calculated from the 3T MRI instead of standard radiographs.

2.8.2.1. MRI Scanning Protocols

3T MRI Scanning Protocol

Animals were scanned on the Siemens 3-T Skyra scanner, using the whole-body coil as the transmitter with a combination of an 18-channel flexible body coil and spine coil as the receivers. After a localizer scan, sagittal T2 images were acquired according to the following sequence parameters:

1) *Sagittal T2-weighted imaging:* TR/TE = 4000/103ms, NEX = 4, FOV = $380 \times 190mm^2$, data matrix = 512 x 205, 28 slices with slice thickness = 1.5mm, voxel size = $0.7 \times 0.7 \times 1.5mm^3$, Flip angle = 146 degree.

9.4T MRI Scanning Protocol

Explanted lumbar spines were also scanned on the Agilent 9.4T Preclinical scanner. Localisation was performed with a T1 scout, the sample was then moved to position the relevant disc for imaging in the centre of the field and each disc was scanned separately in the axial plane according to the following sequence parameters:

1) *Gems-T1 weighted imaging:* TR/TE: 200/4.69ms, FOV: 50x50mm^2, data matrix: 192x192, slice thickness: 0.5, Slice: 10/11, FA: 40, Ave: 4, Dum: 5, PE Rewind, Gradient spoil

2) *Fsems-T2:* TR: 3000ms, FOV:50x50mm^2, data matrix: 192x192, slice thickness 0.5, ESP:8.22, Kzero:5, Ave:2, rep: 1,

2.8.2.2. Disc Height Index

Using standardized methods[7], disc height index (DHI) measurements were calculated and recorded by an observer blinded to the treatment regimen, using standard digital processing software (Osiris MD v8.0.2, Pixmeo, Geneva, Switzerland) based on 3T MRI T2 sagittal images. In this method the average intervertebral disc height was calculated by averaging the measurements obtained from the anterior, middle, and posterior portion of the intervertebral disc and dividing that by the average of the adjacent vertebral bodies.

Following calculation of DHI for each spinal level (L1/2, L2/3, L3/4 and L4/5), absolute change in DHI and percentage change in DHI were calculated over each time point using Microsoft Excel for Mac 15.33 software (Microsoft Corporation, Redmond, WA, USA). Individual DHI scores were then allocated into groups of actual treatment received and analysed using Kruskal-Wallis test of median values followed by Dunn's multiple comparison test. Treated groups were compared using the two-tailed Student t-test followed by Mann-Whitney U-tests using Prism 7.0c for Mac (GraphPad Software Inc., La Jolla, CA, USA).

2.8.2.3. Qualitative MRI Assessment

Sagittal 3T T2 MRI images were scored by three blinded observers using the Pfirrmann disc degeneration classification[8]. Axial 9.4T T2 MRI images were scored for all criteria except intervertebral disc height, which was scored on images reconstructed in the sagittal plane. The classification system is presented below in Table 1.

Table 1 MRI scoring criteria using Pfirrmann classification(8)

Grade	Structure	Distinction of Nucleus and Annulus	Signal Intensity	Height of Intervertebral Disc
I	Homogenous, bright white	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
II	Inhomogeneous with or without horizontal bands	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
III	Inhomogenous, gray	Unclear	Intermediate	Normal to slight decreased
IV	Inhomogenous, gray to black	Lost	Intermediate to hypointense	Normal to moderately decreased
V	Inhomogenous, black	Lost	Hypointense	Collapsed disc space

2.9. Disc Morphology Assessment

Lumbar spinal disc segments allocated for gross morphological and biochemical analysis were sectioned in the horizontal (axial) plane using a 100.0 x 25.0 x 2.5mm blade to provide two complementary halves of the disc as shown in Figure 2. High resolution digital photographs were taken of the exposed complementary surfaces and each region as shown in Figure 2. These digital images were then scored by two blinded observers (CD, PG) using the scoring system shown in Table 2, that was adapted from the method described by Oehme et al. (9) and reported in Daly et al. (10).
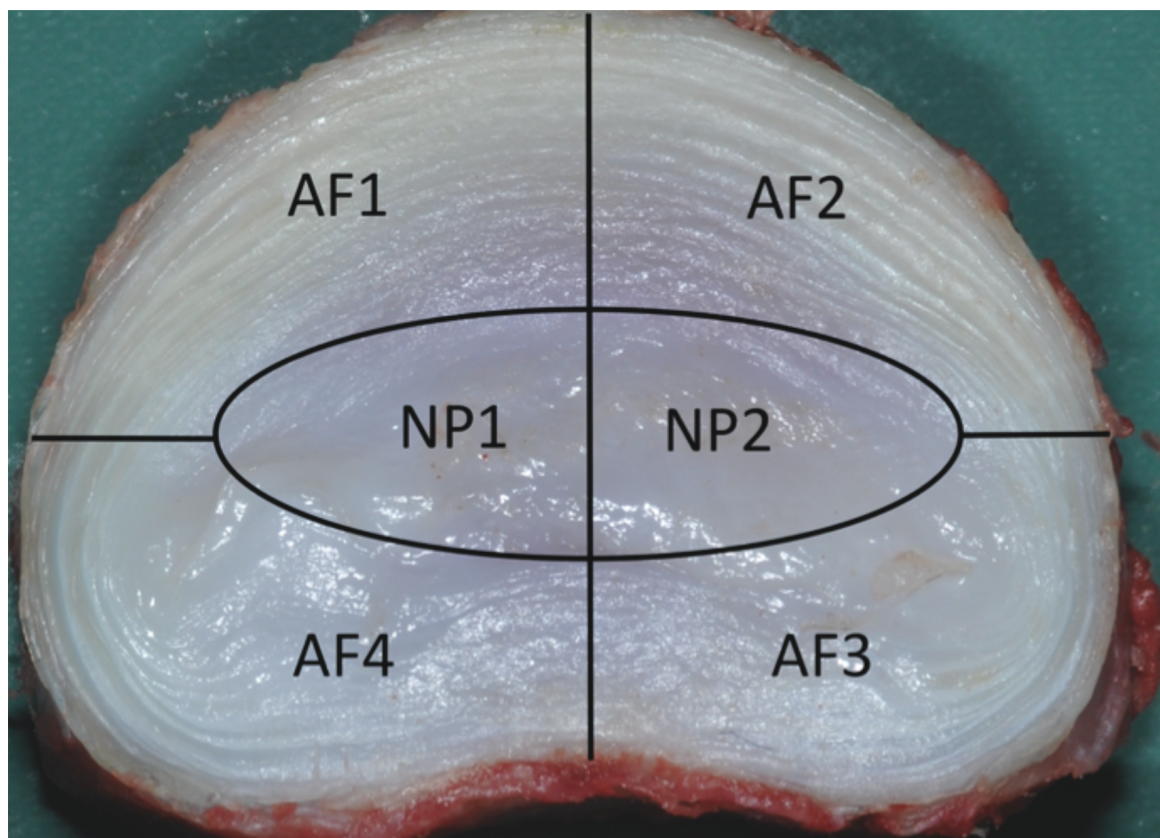
Figure 2. Diagram showing the intervertebral disc segments used for gross morphological and biochemical analysis. AF1 is the site of intervertebral disc annulotomy injury. NP1 is the region of NP on the injured half of the intervertebral disc. NP2 is the half contralateral to the injury. Diagram is taken from Daly et al.(10).

Table 2. Gross morphology criteria used to score AF and NP segmental regions shown in Figure 2 for each disc*

AF Morphological grades applied to each AF quadrant (AF1, AF2, AF3 & AF4)	NP Morphological grades applied to each half (NP1 & NP2) of NP.
Grade 0: Normal Disc Normal disc, no annular disruption, discoloration or hemorrhage.	**Grade 0: Normal NP** No discoloration or hemorrhage
Grade 1: Minor Disruption Annular disruption with minor discoloration and/or hemorrhage	**Grade 1: Minor Disruption** Minor disruption, discoloration and/or hemorrhage. <10% NP region. Minor fissuring and nuclear dehydration may be evident.

Grade 2: Moderate Disruption	Grade 2: Moderate Disruption
Annular disruption with medium discoloration and or hemorrhage.	Medium disruption, discoloration and/or hemorrhage. 10-50% of NP region. Moderate fissuring and nuclear dehydration may be evident.
Grade 3: Major Disruption	Grade 3: Major Disruption
Annular disruption with significant discoloration and/or hemorrhage.	Significant disruption, discoloration and/or hemorrhage. 50-75% NP region. Major fissuring and nuclear dehydration may be evident.
Grade 4: Complete Disruption	Grade 4: Complete Disruption
Annular disruption with extensive discoloration and/or hemorrhage.	Extensive disruption, discoloration and/or hemorrhage. >75% NP region. Extensive fissuring and dehydration may be evident.

*The sum of all regional scores (AF1, AF2, AF3, AF4, NP1 and NP2) yielded a total disc degeneration score between 0 (normal) and 24 (severely degenerated) for each disc. (AF=Annnulus Fibrosis, NP=nucleus pulposus). Table is adapted from the method described by Oehme et al.(9) and taken from Daly et al.(10).

2.10. Biochemical Analysis

2.10.1. Preparation of Tissue for Biochemical Analysis

Following collection of the digital images for morphological assessment, discs from each lumbar spinal level were subjected to biochemical analysis. Specific disc regions utilised in the morphological assessment, and outlined in Figure 1, were analysed separately. The individual nucleus pulposus (NP) and annulus fibrosus (AF) were separated from each other and their vertebral attachments by careful dissection following the boundaries shown in Figure 1. Tissues from each region were finely diced, frozen in liquid N2 and powdered in a liquid Nitrogen cooled ball-mill. The powdered tissues were transferred to pre-weighed Eppendorf vials and weighed, lyophilised and then reweighed to constant weight to determine their anhydrous weights. Aliquots (in triplicate) of the dehydrated tissues were subsequently solubilized using a papain digestion buffer (50 mM sodium acetate [pH = 6.0]) containing 2mg/ml papain (Sigma-Aldrich Chemicals, Sydney, NSW, Australia) by incubation at 60°C for 16 hours (11). The digested tissues were then centrifuged at 3000g for 15 minutes and supernatants diluted to a standard volume (the stock digest solution). Aliquots of the stock solution were analysed for sulphated glycosaminoglycan content (S-GAG) (an index of proteoglycan content) using the dimethylmethylene blue (DMMB) assay(12), hydroxyproline assay (to derive collagen content)(13) and Hoechst dye 33258

assay for DNA content(14) as an index of cell numbers as described below. The results of biochemical analyses were expressed as percentage of tissue dry weight for S-GAG, collagen and DNA.

2.10.2. Determination of Tissue Sulphated Glycosaminoglycan Content

The assay used to quantify S-GAG content described by Farndale et al.(12) was adapted for use in microtitre plates and a plate reader. Chondroitin-6-sulfate (Sigma-Aldrich Chemical, Sydney, Australia) was the standard used. Triplicate amounts of freeze dried disc tissues (2-4 mg) were digested with Papain buffer (50 mM sodium acetate (pH=6.0) containing 2 mg/mL papain (Sigma-Aldrich Chemical, Sydney, Australia) for 16 hours at 60°C(10). The sGAG concentration in diluted aliquots of the digested samples was obtained from the Chondroitin-6-sulfate standard solution diluted in the same papain buffer using the plate reader software.

2.10.3. Determination of Tissue Collagen Content

Triplicate aliquots of the papain digested tissues were hydrolysed in 6 M HCl for 16 hours at 110°C to release the hydroxyproline. The method described by Stegemann and Stalder(13) was used to determine the hydroxyproline content. Hydroxyproline values were multiplied by a factor of 7.4 to provide an index of the tissue collagen concentration.

2.10.4. Determination of Tissue DNA Content

The method of Kim et al.(14) was followed to quantify disc tissue DNA content. Triplicate samples of the papain digested tissues (prepared as described for sGAG analysis) were made up to known stock volumes with 50 mM Tris buffer containing 0.01M NaCl. Triplicate aliquots were pipetted into the wells of a microtitre plate followed by the addition of the Hoechst 33258 reagent (Sigma-Aldrich Chemicals, Sydney, Australia) also dissolved in Tris buffer/NaCl buffer pH 7.2. Fluorescence intensity was determined immediately by its emission at 450 nm (excitation at 350 nm) using a microtitre plate reader (Flouroskan II)(Thermo Fisher Scientific Australia, Scoresby, VIC, Australia).

2.11. Histological Analysis

2.11.1. Histological Preparation

Harvested individual disc segments, consisting of the intervertebral disc with the sawn vertebral bodies attached, were placed in 10% neutral buffered formalin for eight days and then transferred to 70% ethanol for storage. The volume of vertebral bone was reduced down to the growth plate using a fine diamond saw. Prior to paraffin based tissue embedding, decalcification of the remaining vertebral bone was undertaken with multiple changes of 10% formic acid. Coronal paraffin sections of the entire disc segments, of the discectomy annulotomy injured and half of the control discs, were cut using a standard rotary microtome and stained using H & E, and Alcian Blue/Picrosirus red employing standard

protocols. The drill injured discs and remaining control discs were sectioned in an axial plane, in order to demonstrate the entire injury tract and adjacent tissue of the drill injury.

As a result of the extended time required to completely decalcify the vertebral bone, coupled with marked disruption of the AF tissue integrity in the surgical zone, the quality of many of the disc tissue sections were considered unacceptable for quantitative histological scoring. Nevertheless, all histological sections were reviewed qualitatively. Images were analysed using Abrio software (CRI, Woburn, USA) that allowed additional qualitative assessments between the experimental groups to be determined.

2.11.2. Birefringent Microscopic Analysis

H & E sections from the specimens of the study described in Chapter 4 were examined by polarised light microscopy to identify changes of AF collagen fibre orientation using birefringence. Images were captured using a CCD camera mounted on a Leica DMIRB base microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

2.12. Online Survey

A survey of Australasian Neurosurgeons was conducted by email invitation sent to all full members of the Neurosurgical Society of Australasia. The survey consisted of 11 multi-choice questions conducted by an anonymized online survey. The survey was administered electronically via SurveyMonkey (SurveyMonkey, San Mateo, CA, USA).

The survey questions are listed below:

Table 3. Survey questions and possible responses. The table lists all survey questions and possible responses (from Daly et al. (15))

Question	Response
1. Which surgical technique do you perform as a standard procedure for lumbar diskectomy?	Bilateral muscle dissection with bilateral diskectomy
	Bilateral muscle dissection with unilateral diskectomy
	Unilateral muscle dissection with unilateral diskectomy
	Unilateral muscle dissection with unilateral diskectomy via tubular system
	Bilateral muscle dissection with bilateral diskectomy
2. Do you use magnification when performing lumbar diskectomy operations and if so what kind?	No magnification
	Loupes
	Microscope
3. In the absence of cauda equina syndrome or severe neurological deficit, what is the minimum duration of radicular pain a patient must report for you to offer lumbar diskectomy surgery?	<2 weeks
	2-4 weeks
	4-8 weeks
	8-12 weeks
	>12 weeks
4. In the absence of CSF leak when do you allow your lumbar diskectomy patients to mobilize following their operation?	Day 0, upon returning to the ward
	Day 0, after a few hours
	Day 1
	Day 2
5. Do you prescribe inpatient post-operative physiotherapy during admission following lumbar diskectomy?	Never
	Rarely
	Sometime

	Often
	Always
6. Do you prescribe postoperative outpatient physiotherapy after diskharge following lumbar diskectomy?	Never
	Rarely
	Sometime
	Often
	Always
7. Do you advise sitting restrictions in the post-operative period following lumbar diskectomy?	No sitting restrictions
	As comfort allows
	<30 mins per hour
	<15 mins per hour
	Avoid sitting entirely if possible
8. How long after lumbar diskectomy do you advise patients to restrict sitting time?	I don't advise sitting restrictions
	Until comfortable
	<1 week
	1-2 weeks
	2-4 weeks
	4-8 weeks
	>8 weeks
9. Do you advise lifting restrictions in the post-operative period?	I don't advise lifting restrictions
	<40kg
	<20kg
	<10kg
	<5kg

10. How long after microdiskectomy do you advise patients to restrict lifting?	No lifting restrictions
	Until comfortable
	<1 week
	2-4 weeks
	4-8 weeks
	>8 weeks
11. How many years have you been practicing as a Consultant Neurosurgeon?	0-5 years
	6-10 years
	11-15 years
	16-20 years
	>20 years

2.13. Statistical Analysis

All data analysis and storage was performed using Prism 7.0c for Mac (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft Excel for Mac (Version 15.33, Microsoft, Redmond, WA, USA). Parametric data were analysed using one-way ANOVA, and the Tukey's multiple comparison test was performed when significant differences in means were observed. Nonparametric data were analysed using Kruskal-Wallis test of median values followed by Dunn's multiple comparison test. Treated groups were compared using the two-tailed Student t-test followed by Mann-Whitney U-tests. Pearson chi-squared statistic was used to assess the significance of relationships between surgeon seniority and response to variables in the survey of Australasian Neurosurgeons addressing lumbar discectomy peri-operative practices. A p value < 0.05 was considered statistically significant.

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Chapter 3.   A Comparison of Two Ovine Lumbar Intervertebral Disc Injury Models for the Evaluation and Development of Novel Regenerative Therapies

3.1.   Introduction

This chapter describes a comparison of two ovine models of the post lumbar discectomy intervertebral disc for consideration for use in the subsequent investigation of pentosan polysulfate primed mesenchymal progenitor cells for intervertebral disc regeneration. This chapter contains the manuscript for an experimental study entitled, **"A Comparison of Two Ovine Lumbar Intervertebral Disc Injury Models for the Evaluation and Development of Novel Regenerative Therapies."** This manuscript has been submitted to The Global Spine Journal, and is currently under review.

The candidate, Chris Daly, contributed to the experimental design, experimental procedures and the writing of the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xvii.

A Comparison of Two Ovine Lumbar Intervertebral Disc Injury Models for the Evaluation and Development of Novel Regenerative Therapies

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A Comparison of Two Ovine Lumbar Intervertebral Disc Injury Models for the Evaluation and Development of Novel Regenerative Therapies

Abstract:	Study Design Large animal research Objective Lumbar discectomy is the most commonly performed spinal surgical procedure. We investigated two large animal models of lumbar discectomy in order to study the regenerative capacity of mesenchymal stem cells following disc injury. Methods: Twelve adult ewes underwent baseline 3T MRI followed by lumbar intervertebral disc injury by either drill bit (n = 6) or annulotomy (n = 6). Necropsies were performed six months later. Lumbar spines underwent 3T and 9.4T MRI prior to histological, morphological and biochemical analysis. Results: Drill bit injured (DBI) and annulotomy injured discs demonstrated increased Pfirrmann grades relative to uninjured controls (p<0.005), with no difference between the two models. Disc height index loss was greater in the annulotomy group compared to the DBI group (p<0.005). Gross morphology injury scores were higher in annulotomy than DBI discs (p < 0.05) and both were higher than controls (p < 0.005). Proteoglycan content was reduced in the discs of both injury models relative to controls (p<0.005), but lower in the annulotomy group (p < 0.05). Total collagen content of the annulotomy group disc regions were higher than DBI and control discs (p<0.05). Histology revealed more matrix degeneration, vascular infiltration and granulation tissue in the annulotomy model. Conclusion: Although both models produced disc degeneration, the annulotomy model better replicated the pathobiology of human discs post discectomy. We therefore concluded that the annulotomy model was a more appropriate model for the investigation of the regenerative capacity of mesenchymal stem cells administered post discectomy.

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Abstract

Study Design

Large animal research

Objective

Lumbar discectomy is the most commonly performed spinal surgical procedure. We
investigated two large animal models of lumbar discectomy in order to study the
regenerative capacity of mesenchymal stem cells following disc injury.

Methods:

Twelve adult ewes underwent baseline 3T MRI followed by lumbar intervertebral disc
injury by either drill bit (n = 6) or annulotomy (n = 6). Necropsies were performed six
months later. Lumbar spines underwent 3T and 9.4T MRI prior to histological,
morphological and biochemical analysis.

Results:

Drill bit injured (DBI) and annulotomy injured discs demonstrated increased Pfirrmann
grades relative to uninjured controls (p<0.005), with no difference between the two models.
Disc height index loss was greater in the annulotomy group compared to the DBI group

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(p<0.005). Gross morphology injury scores were higher in annulotomy than DBI discs (p < 0.05) and both were higher than controls (p < 0.005). Proteoglycan content was reduced in the discs of both injury models relative to controls (p<0.005), but lower in the annulotomy group (p < 0.05). Total collagen content of the annulotomy group disc regions were higher than DBI and control discs (p<0.05). Histology revealed more matrix degeneration, vascular infiltration and granulation tissue in the annulotomy model.

Conclusion:

Although both models produced disc degeneration, the annulotomy model better replicated the pathobiology of human discs post discectomy. We therefore concluded that the annulotomy model was a more appropriate model for the investigation of the regenerative capacity of mesenchymal stem cells administered post discectomy.

Keywords:

Animal model

Intervertebral disc

Discectomy

Regeneration

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Introduction

Lower back pain causes more global disability than any other condition worldwide[1]. Lower back pain commonly results from degenerative lumbar disc disease causing discogenic pain [2]. Lumbar disc degeneration is a complex process manifested by changes in cellular, matrix, endplate and the neurovascular components of the intervertebral disc. Intervertebral disc herniation is a common outcome of lumbar disc degeneration, whilst lumbar discectomy is the most commonly performed spinal surgical procedure[3]. Lumbar discectomy successfully treats radicular symptoms associated with neural compression in over 80% of patients[4]. However, the procedure fails to address the underlying pathophysiology of intervertebral disc degeneration responsible for the syndrome. Moreover, following lumbar discectomy up to one third of patients report low back pain[5]. In addition, up to 18% of patients experience recurrent disc herniation[6] with 12% undergoing reoperation within four years[7]. Ultimately 40% of these patients will undergo spinal fusion[7].

Given the significant disease burden resulting from disc degeneration and lower back pain numerous animal models have been developed to further understand the pathobiology of disc degeneration and examine potential modalities for its treatment[8]. There are, however,

relatively few reports of large animal models of lumbar discectomy[9-12]. Given the clinical

ubiquity of discectomy, the inherent anatomical challenges to disc repair and the

opportunity presented to initiate regenerative therapy at the time of surgical intervention,

we sought to develop a suitable large animal model of discectomy that could be used to

evaluate potential tissue regenerative therapies, such as transplantation of stem cells.

Limited annular injury to ovine discs have been widely used to generate a model of disc

degeneration[13-15]. The ovine species has also been used to test implant devices and in the

preclinical investigation of cellular therapies to support spinal fusion and disc reconstitution

[9,16-18]. Ovine discs, like human discs, undergo chondroid metaplasia with skeletal

maturation[19], due to the loss of their notochordal cell remnants[19,20]. Additionally, the ovine

disc is closer in size and cellular phenotype[20] to the human intervertebral disc than small

animal models, important characteristics given the nutritional limitations associated with of

the central regions of the disc. Furthermore, despite its quadrupedal conformation, the

sheep spine has been shown to exhibit significant biomechanical similarities to the human

spine[21].

We have previously described the use of a modified ovine annulotomy model for investigation of the potential of mesenchymal progenitor cells (MPCs) formulated with the pharmaceutical agent, Pentosan Polysulfate (PPS), embedded in a biodegradable gelatin scaffold to promote intervertebral disc regeneration following lumbar discectomy in a pilot study[9]. In this modified annulotomy model a full thickness 3 x 5mm annulotomy was performed with a scalpel blade and 200mg of annular and nuclear tissue removed with a pituitary rongeur. PPS was used as it was known to enhance MPC viability and promote their differentiation to a chondrogenic phenotype whilst also inhibiting osteogenesis[22]. Our pilot study demonstrated the feasibility of the modified annulotomy model and provided positive outcomes on the efficacy of the MPC+PPS formulation. However, prior to further investigations of other potential therapeutic modalities of lumbar disc repair that required the use of a liquid hydrogel, we sought to determine the most appropriate large animal model for such applications.

An earlier publication by Zhang et al.[23] reported that disc degeneration could be induced in goat lumbar discs by using a drill-bit to penetrate the AF through to the NP. Using a subjective histological grading system this model was reported to provide more reliable degenerative changes than insertion of a horizontal surgical blade along the same path. In

principle the Zhang et al.[23] model offers advantages in facilitating the injection of

regenerative liquid hydrogels/cell combinations into the disc without the use of a solid

scaffold, which was a requirement of using the Oehme discectomy model[9]. However, the

study of Zhang et al.[23] was performed in goats and did not include biochemical analysis of

the injured intervertebral discs, thereby limiting the ability to directly compare these two

models directly.

In the present study, we evaluated the annulotomy and drill-bit methods of surgically

inducing disc failure using a homogeneous group of adult sheep and monitoring the relative

outcomes six months later using both subjective and objective methods of assessment.

Material and methods

Surgical procedure

With ethics approval from the Monash Medical Centre Animal Ethics Committee and

conforming to the Australian code of practice for the care and use of animals for scientific

purposes (8th edition, 2013), 12 adult (two to four years of age) Border-Leicester Merino

cross-bred ewes underwent preoperative 3 Tesla MRI (Siemens Skyra Widebore 3T MRI,

Siemens, Erlangen, Germany) under general anesthetic. Ewes were used in this study due

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to their better temperament than males- castrate or intact. Sheep were raised in open

pastures and ambulated freely prior to the trial. All sheep were fasted for 24 hours prior to

surgery and anesthetized using intravenous thiopentone (10-15mg/kg) (Bayer Australia

Ltd., Pymble, NSW, Australia) followed by intubation and isoflurane inhalation

(Pharmachem, Eagle Farm, QLD, Australia) (2-3% in oxygen).   Sheep were placed in the

right lateral position. Local anesthetic (bupivacaine 0.5%)(AstraZeneca Australia,

Macquarie Park, NSW, Australia) was administered subcutaneously and the L2-3 and L3-4

lumbar intervertebral discs exposed via left lateral retroperitoneal approach, as previously

described [24,25]. Intraoperative lateral radiographs (Radlink, Atomscope HF200A, Redondo

Beach, CA, USA) were performed to confirm the correct levels. Six sheep underwent

microdiscectomy annulotomy injury, performed by the creation of a 3mm x 5mm annular

window followed by disc resection using pituitary rongeurs. The disc tissues collected

(200.0 +/- 3.0 mg) consisted mainly of annulus fibrosus (AF) with some nucleus pulposus

(NP). The adjacent L1-2 and L4-5 discs served as untreated controls. Drill bit injury was

performed on the L2/3 and L3/4 intervertebral discs of the remaining six sheep using a

3.5mm Brad point drill bit (Carbatec, Melbourne, Victoria, Australia) with a drill bit stop

applied at 12mm drill bit length (Drill Warehouse, Amazon, Seattle, USA) as described

previously[25].

Following intervertebral disc injury the wound was closed using a routine layered

procedure performed using absorbable sutures (Vicryl, Ethicon, NJ, USA). Animals

received a fentanyl patch (Duragesic 75 µg/hr, Jannsen LLC., North Ryde, NSW, Australia)

and intravenous paracetamol (Pfizer Ltd., West Ryde, NSW, Australia) for postoperative

analgesia. Following recovery, animals were returned to the pen with other sheep and

allowed free ambulation. Sheep were returned to open pasture one week post-surgery.

Necropsy

Six months post-surgery animals were euthanized by intravenous injection of 150 mg/kg of

pentobarbital (Sigma-Aldrich, Castle Hill, NSW, Australia). The lumbar spines were then

removed *en-bloc*, a segment was isolated from the mid-sacrum to the thoracolumbar

junction and transferred to Monash Biomedical Imaging for MRI analysis. Spinal columns

were then transected in the horizontal plane through their vertebral bodies, using a band

saw, to provide spinal segments consisting of a complete lumbar disc with half of the

adjacent vertebral bodies attached. Subsequent gross morphological, biochemical and

histological analysis of discs were undertaken using these spinal segments as described

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below. Spinal segments containing discs destined for histological analysis were transferred

to phosphate buffered formalin.

Radiological Analysis

Using standardized methods, disc height index (DHI) measurements were calculated and

recorded by an observer blinded to the treatment regimen, using standard digital processing

software (Osiris MD v8.0.2, Pixmeo, Geneva, Switzerland).

Sagittal 3T (Siemens Skyra Widebore 3T MRI, Siemens, Erlangen, Germany) T2 weighted

MRI sequences of the entire lumbar spine explant were obtained for each animal. Axial

9.4T (Agilent 9.4T MRI Small Animal Scanner Agilent/Varian, Santa Clara, CA, USA) T1

and T2 MRI sequences of the control and intervention lumbar intervertebral discs were

taken for each animal. Using sagittal 3T T2-weighted sequences and 9.4T T2 sagittal

reconstructions (Osiris MD v 8.0.2) four blinded observers (a neuroradiologist,

neurosurgeon and two neurosurgery residents blinded to the treatment regimen) determined

the Pfirrmann MRI disc degeneration scores for all lumbar discs.

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Disc height index analysis was also performed using the pre-operative and 3T MRI images

obtained at necropsy, by an observer blinded to the intervention protocol. The 3T MRI

assessment of the disc height index eliminated the potential for parallax error while also

producing consistent image quality for all discs.

Gross Morphological Analysis

Lumbar spinal disc segments allocated for gross morphological and biochemical analysis

were sectioned in the horizontal (axial) plane using a 100.0 x 25.0 x 2.5 mm blade to

provide two complementary halves of the disc as shown diagrammatically in **Fig. 1**. High

resolution digital photographs were taken of the exposed complementary surfaces and each

region shown in **Fig. 1** scored by a blinded observer following the criteria in **Table 1**

described by Daly et al.[26]   and adapted from the method of Oehme et al.[27]

Biochemical Analysis

After collection of the digital images of discs for morphological analysis all tissue regions

shown in Fig. 1 were subjected to biochemical analysis. The individual annulus fibrosus

(AF) and nucleus pulposus (NP) from each region were separated from each other and their

vertebral attachments by careful dissection using the boundaries shown in Fig. 1. Tissues

from each region were finely diced, frozen in liquid N2 and powdered in a liquid Nitrogen cooled ball-mill. The powdered tissues were transferred to pre-weighed Eppendorf vials and weighed, lyophilised and reweighed to constant weight to determine their anhydrous weights. Triplicate aliquots of the dehydrated tissues were solubilized using a papain digestion buffer (50 mM sodium acetate {pH = 6.0]) containing 2mg/ml papain (Sigma-Aldrich Chemicals, Sydney, NSW, Australia) by incubation at 60°C for 16 hours [28]. The digested tissues were centrifuged at 3000g for 15 minutes and supernatants diluted to standard volumes (the stock digest solution). Aliquots of the stock solution were analysed for sulphated glycosaminoglycan (S-GAG) (an index of proteoglycan content) levels using the dimethylmethylene blue (DMMB) assay[29] and hydroxyproline assay (to derive collagen content)[30]. The results of biochemical analyses were normalized and were expressed as percentage of tissue dry weight for S-GAG and collagen.

Histological Analysis

The individual disc segments, consisting of the intervertebral disc with attached hemisected vertebral bodies were in 10% neutral buffered formalin for eight days then stored in 70% ethanol. The volume of vertebral bone was reduced to the growth plate using a fine diamond saw. Prior to paraffin based tissue embedding, decalcification of the remaining

vertebral bone was undertaken with multiple changes of 10% formic acid. Coronal paraffin

sections of the entire disc segments for the annulotomy sheep and axial sections for the drill

injured sheep were cut using a standard rotary microtome and stained using H & E. Axial

sections were taken from the drill injury disc to allow for visualization of the entire drill bit

injury tract. The annulotomy injured disc were only subjected to standard coronal plane

sectioning.

Statistical Analysis

All data analysis and storage was performed using Microsoft Excel for Mac (Version 15.33,

Microsoft, Redmond, WA, USA) and Prism 7.0c for Mac (GraphPad Software Inc., La

Jolla, CA, USA). Parametric data were analyzed using one-way ANOVA, and the Tukey's

multiple comparison test was performed when significant differences in means were

observed. Nonparametric data were analyzed using Kruskal-Wallis test of median values

followed by Dunn's multiple comparison test. Groups were compared using the two-tailed

Student t-test followed by Mann-Whitney U-tests.   A p value < 0.05 was considered

statistically significant.

Results

Disc Height Index

The annulotomy injured discs demonstrated significantly greater loss of height than the drill injured and control discs (p<0.005). However, both the annulotomy and drill injured discs demonstrated greater loss of height than control discs (p<0.005) (**Fig. 2A**).

Baseline pre-operative 3T MRIs of all animals revealed no evidence of underlying disc degeneration at control or intervention levels (L1/2 to L4/5). Scoring of drill bit and annulotomy injured discs demonstrated significantly increased Pfirrmann grades relative to control discs (both p<0.005), however there was no significant difference in Pfirrmann grades between the two injury groups (**Fig. 2A, 2B & 2D**).

9.4 T MRI

The horizontal images obtained by 9.4T MRI allowed ready appreciation of the extent of annular disruption observed in the annulotomy vs. drill injured intervertebral discs (**Fig. 3B & C**) and correlated well with gross morphological observations. However, the 9.4T MRI

Pfirrmann grades were consistent with 3T grades once sagittal reconstruction and grading

was performed (**Fig. 2B and 3D)**. Significantly increased Pfirrmann grades were observed

in the drill and annulotomy injured discs compared to control discs ($p<0.005$) with no

significant difference between the two injury models.

Gross Morphology

Gross morphological analysis was completed on a regional basis, with the data presented in

Figure 4D showing the aggregate morphological scores. Representative gross

morphological digital images are displayed in **Fig. 4A, B & C** and their respective scores,

determined using the criteria described in Table 1 and shown in Fig4D. Drill injured discs

generally demonstrated a more focal annular and nuclear tract injury(**Fig. 4B),** with

minimal nucleus pulposus disorganization, in comparison to the annulotomy injured discs,

which showed more widespread changes and blood degradation product staining (**Fig. 4C**).

Nonetheless, total disc gross morphological scores were significantly increased in both the

drill bit injured and annulotomy injured intervertebral discs relative to control ($p<0.005$).

Annulotomy injured intervertebral disc gross morphological scores were, however,

significantly elevated relative to drill injured discs ($p<0.05$)(**Fig. 4D**).

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Proteoglycan content as determined by Sulfated-Glycosaminoglycan (S-GAG) analysis

S-GAG content was significantly reduced in the injured (NP1) (**Fig. 5A**) and contralateral

NP region (NP2) (**Fig. 5B**) in both the drill and annulotomy injured discs relative to

controls ($p<0.005$). Furthermore, the annulotomy discs demonstrated significantly less

NP1 S-GAG and NP2 S-GAG than the drill injured discs ($p<0.005$ and $p<0.05$

respectively). This relationship persisted for NP Total S-GAG content (**Fig. 5C**). Total

Disc S-GAG content demonstrated a significant reduction in S-GAG content in the

annulotomy injured disc compared to both control ($p<0.005$) and drill injured discs

($p<0.05$) (**Fig. 5D**). There was no significant difference between control and drill injured

total disc S-GAG content.

Collagen Content

The collagen content of the annulotomy injury site AF (AF1) was significantly higher than

both the control and drill injured discs ($p<0.05$) (**Fig. 6A**). Furthermore, the drill injury

AF1 collagen content was significantly lower than control discs ($p<0.05$). The annulus

fibrosus adjacent to the injury site (AF4) also demonstrated a significant increase in

collagen content in the annulotomy group relative to controls ($p<0.01$) (**Fig. 6B**). The

nucleus pulposus, both ipsilateral and contralateral to the injury site, demonstrated

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significantly higher collagen content in the annulotomy injured disc relative to both the

control and drill injured discs (p<0.005 and p<0.05 respectively) (**Fig. 6C & D**). These

differences were also reflected in the total NP and total disc (NP +AF) collagen content of

the annulotomy group compared to both the drill injury and control discs (**Figs. 6E & F)**

(p<0.001 and p<0.005 respectively).

Histology

Qualititative histological analysis revealed differences between the injury models with

regard to the disc ultrastructure and extent of vascular infiltration and granulation tissue

deposition. Control discs demonstrate intact annulus fibrosi displaying multiple lamellae

abutting the proteoglycan rich nucleus pulposus (**Fig. 7A & 8A [axial] & 7 B & 8B**

[coronal]). Axial H & E stained slides of drill injured disc demonstrate the focal nature of

the disruption of AF and NP tissues induced by this injury model (**Fig. 7C and 7D)**.

Relatively little vascular infiltration is evident. Reduced proteoglycan content is evident on

the drill injured disc (**Fig. 8C)** relative to the control disc **(Fig. 8A)** on the Safranin O Fast

Green stained slides. The annulotomy injured disc (**Fig. 7E)** demonstrates extensive

disruption of lamellae with deposition of granulation tissue and infiltration within the injury

site. High power microscope images highlighted the extent of vascular proliferation

occuring at the injury site of the AF (**Fig. 7F).** Marked reduction in proteolgycan content is also evident at the annulotomy injury site (**Fig. 8D)**.

Discussion

The results of the present study demonstrated that both the modified annulotomy injury model originally described by Oehme et. al.[9] and a modification of the drill injury model described by Zhang et. al.[23] induced degeneration in sheep lumbar discs six months following the surgical intervention. Furthermore, as assessed from the disc height indices, gross morphological, biochemical and histological analyses, the annulotomy injury provoked more extensive degenerative changes in injured discs, than observed with the drill injury procedure.

We suggest that the extent and nature of the degenerative changes induced in the discs of each model was determined by the relative magnitude of several time dependent mechanical and biological events that are known to dictate the pathology of disc degeneration[31-36]. For example, surgical compromise of AF hoop stress tensile function and reduction in NP hydroelasticity would impose an immediate disturbance in the nature of the

mechanical stresses acting across the spinal unit and its adjacent structures[37]. A secondary event elicited, would be an inflammation and an early repair response at the site of injury, with proliferation of the capillaries serving the outer AF, accompanied by deposition of granulation and fibrotic tissue within the defect site. Such events have been described in other animal models[38-41] and are the sequellae of human disc herniation[42].

Loss of proteoglycan and its associated water is a relatively early event in the pathophysiology of intervertebral disc degeneration[31,43]. The loss of these matrix components leads to marked changes in the mechanical properties of the intervertebral disc and adjacent structures, as intradiscal pressure is reduced and the ability of the disc to tolerate compressive loading diminishes[44]. Biomechanical studies have demonstrated such changes in the mechanical behavior of discs that were proportional to the size of the annular defects[35,37,45].

The compromise of AF integrity and loss of NP material initiated in the annulotomy model closely mirrors the clinical condition of symptomatic lumbar intervertebral disc herniation. In the clinical setting, radicular symptoms are caused by herniated intervertebral disc material producing neural compression. This herniated AF and NP, and any additional

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loose NP, is excised during conventional lumbar microdiscectomy. The creation of a full thickness annulotomy, with removal of 200mg of annular and nuclear tissue, differentiates this modified ovine annulotomy model from those recently reported by Shu et al.[46] and most other ovine annular injury models in the literature[13,14] in which partial thickness annular incisions are performed. Such models may be more representative of spontaneous intervertebral disc degeneration, in which annular tears are often observed, than the post lumbar discectomy intervertebral disc.

The model of Zhang et al.[23], demonstrated the ability of the drill bit injury to induce disc degeneration two months following surgery, confirmed histologically but not biochemically. Furthermore, the degeneration observed histologically did not produce correlative MRI changes such as increased 1.5T MRI Pfirrmann grades. Many prior studies of the intervertebral disc, including that of Zhang et al.[23], were performed with 1.5T MRI scanners. With the advance of technology 3T MRI scanners are commonly available in the clinical setting and the 9.4T MRI scanner is now available for preclinical studies. Despite our use of both 3T and 9.4T MRI the MRI Pfirrmann grade findings did not directly parallel our histological, biochemical, morphological and disc height index observations. We suggest the discrepancy between the MRI and our other findings may be attributed to

the relatively low sensitivity of conventional MRI Pfirrmann Grades for detecting subtle

degenerative differences. The 9.4T MRI axial images (**Fig. 3**) provided superior resolution

of anatomical detail compared to conventional 1.5T and 3T MRI scanners[47], however, this

additional data was not captured in the Pfirrmann grading system when the axial images

were converted to the sagittal plane. Advanced quantitative radiological methods, such as

T_{1rho}-weighted MRI, may provide a more sensitive method of measuring early

intervertebral disc degeneration radiologically using the clinical magnets currently

available[48].

The increase in collagen observed in the injury site AF and NP of the annulotomy injured

disc reflects the normal tissue response to traumatic injury, with inflammation, capillary

invasion and fibrotic granulation tissue deposition representing key matrix events. The

repair tissues that normally accumulate at these early injury sites, consist mainly of type I

collagen, and are accompanied by the loss of proteoglycans[33] as was observed for the

annulotomy model but not drill bit model. In healthy discs and the early stages of disc

degeneration, the chondrocyte-like cells of the NP synthesize predominantly type II

collagen[49]. However, as the disc degeneration progresses, these cells undergo a transition

and synthesize type I collagen and less proteoglycans[33] . Therefore, it is possible that the

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endogenous NP cells of the injured discs in the annulotomy model also contributed to the

deposition of fibrotic tissue. Additional studies of the collagen subtypes resident in the

various regions of the injured discs from the annulotomy model are required to resolve this

important question. The observed lack of a significant difference in collagen content

between the drill injured disc and control discs, with the exception of AF1, is indicative of

the relatively low grade degeneration changes induced in these tissues by either surgical

approach.

As a result of the extended time required to completely decalcify the vertebral bone, the

quality of the disc tissue sections were unfortunately unacceptable for quantitative

histological scoring. Nevertheless, all histological sections were reviewed qualitatively.

On histological examination, vascular invasion was noted in the annulotomy injured discs

that was relatively absent from the drill bit injured discs. Vascular invasion is consistent

with histological observations from herniated and degenerate intervertebral discs in the

clinical context[50,51]. In addition, the annulotomy injured discs demonstrated

disorganization of the lamella pattern of the AF and adjacent NP. In contrast, the drill bit

injured disc demonstrated a focal lesion with minimal disruption of the AF and NP. The

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absence of significant tissue ingrowth observed in drill injured specimens is in agreement

with the lack of a significant increase in collagen content in the drill injured AF.

There are, however, important differences between these preclinical models and the human

intervertebral disc which contribute to the limitations of this study. A limitation common

to large animal models of intervertebral disc degeneration is the inability to assess low back

pain. The only animal models allowing for low back pain assessment are rodent

models[52,53]. Such models present challenges in regard to their differences to the human disc

in size, anatomy, the presence of notochordal cells and ultimately translatability.

Furthermore, in the clinical situation, the underlying disc demonstrates pre-existing

degeneration culminating in disc prolapse and neural compression. The models we present

are of normal intervertebral discs subjected to injury to induce intervertebral disc

degeneration. Discs with pre-existing degeneration may have reduced capacity to repair or

regenerate or may demonstrate a higher extent of degeneration following injury induction

than the previously healthy discs included in this trial. This is an important consideration

in the context of investigating regenerative therapies for disc degeneration in the future.

Conclusion

The ovine intervertebral disc drill-bit and annulotomy injury models both produce intervertebral disc degeneration at six months following injury. However, the severity of degeneration associated with the annulotomy model was greater, as assessed by MRI, gross morphology, biochemical and histological analysis, than the drill-bit model. Moreover, the ovine annulotomy injury model better replicated the post discectomy lumbar intervertebral disc mechanistically and importantly more closely reproduced the known pathology of disc tissues examined post human disc herniation[50,51].

On the basis of the aforementioned findings we concluded that the ovine annulotomy model provides a more suitable animal model for the evaluation of novel cellular modalities that targeted disc repair than the drill-bit model. In this regard, we have subsequently utilized the ovine annulotomy model of microdiscectomy to compare the relative efficacy of two mesenchymal stem cell preparations in their ability to restore disc integrity six months post surgery[26].

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<u>Figure Legends</u>

Fig. 1. Diagram showing the Intervertebral disc segments used for gross morphological and biochemical analysis. AF1 is the site of intervertebral disc annulotomy injury. NP1 is the region of NP on the injured half of the intervertebral disc. NP2 the complementary half of NP1. (AF= annulus fibrosus, NP= nucleus pulposus).

Fig. 2A. Necropsy Sagittal MRI of the drill injury lumbar spine and **2B. Annulotomy injured lumbar spine** demonstrating increased Pfirrmann Grade in the injured disc (L2/3 and L3/4) relative to control discs (L1/2 and L4/5). 2C**. Percentage Reduction in Disc Height Index.** Annulotomy and drill injury discs demonstrated significantly greater loss of disc height than control discs**. 2D**. **3T Pfirrmann grades**   Drill bit and annulotomy injured discs had significantly increased Pfirrmann grades relative to control discs however there was no significant difference in Pfirrmann grades between injury groups. (* signifies p<0.05, ** signifies p<0.005).

Fig. 3A 9-4T MRI axial images A. Control disc demonstrating intact AF and hyerintense NP.

B. Drill injury disc demonstrating injury tract (arrow) extending to NP **C. Annulotomy disc** demonstrating broader injury tract (arrow) and decreased NP hyperintensity. **D. 9.4T MRI Pfirrmann grades** Drill injury and annulotomy injured discs demonstrated significantly higher 9.4T Pfirrmann Grades than control discs with no signifcant difference between injured groups. (AF= annulus fibrosus, NP= nucleus pulposus) (* signifies p<0.05, ** signifies p<0.005).

Fig. 4. Representative examples of disc gross morphology. A. Control disc demonstrating intact AF and gelatinous white NP (scored as 0). **B. Drill injury disc** demonstrating injury tract extending to NP (arrow) with discolouration of NP and AF disruption at injury site (scored as 4) C. **Annulotomy disc** demonstrating broader AF injury (arrow) with extension to NP, loss of NP material, discolouration of NP and AF (scored as 6). **D. Gross morphology scores** Drill injury and annulotomy injured discs demonstrated significantly higher gross morphology scores than control discs. Annulotomy discs demonstrated higher gross morphology scores than drill injury discs. (* signifies p<0.05, ** signifies p<0.005).

Fig. 5. Sulfated-GAG content (% dry weight). A. NP1 GAG is significantly lower in annulotomy than drill-injured discs which is in turn less than control discs. **B. NP2** demonstrates the same relationship with annulotomy injured discs lower than drill injured discs ($p<0.05$) which in turn are less than control discs   **C. NP TOTAL GAG** and **D. TOTAL DISC GAG** demonstrates the same series of relationships. (* signifies $p<0.05$, ** signifies $p<0.005$).

Fig. 6. Collagen content (% dry weight). A. AF1 Annulotomy group discs had significantly more collagen than both control and drill injured discs. **B. AF4** Annulotomy group collagen was significantly higher than control discs.   **C. NP-1** Annulotomy group collagen was significantly higher than both control and drill injury collagen.   **D. NP2** collagen demonstrated the same pattern. **E. NP TOTAL** also demonstrated an increase in collagen in the annulotomy group relative to the control group.   **F. TOTAL DISC collagen** was significantly increased in the annulotomy group relative to both control and drill injured discs. (* signifies $p<0.05$, ** signifies $p<0.005$).

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Fig. 7. Sections stained with Hematoxylin & Eosin. A. Control disc (axial section) demonstrating intact annulus fibrosus and adjacent nucleus pulposus. **B. Control disc (coronal section)** demonstrating the coronal plane. **C. Drill injured disc** demonstrating disruption of the annulus fibrous extending into the nucleus. **D. Drill injured disc** under higher power demonstrating superficial localized fibrosis (arrow) at external AF with minimal vascular infiltration. **E. Annulotomy injured disc** demonstrating extensive lamellae disruption and vascular infiltration. **F. Annulotomy injured disc** demonstrating lamellar structure under high power magnification with evidence of marked vascular invasion (arrow). Scale bar = 200 um.

Fig. 8. Sections stained with Safranin O and Fast Green. A. Control disc (axial section) demonstrating intact annulus fibrosus and adjacent nucleus pulposus. **B. Control disc (coronal section)** demonstrating the coronal plane. **C. Drill injured disc** demonstrating reduced Safranin O stain (indicative of reduced proteoglycan content) in the drill injured region of the nucleus pulposus (arrow). **D. Annulotomy injured disc** demonstrating significant reduction in Safranin O stain in the injured region of the intervertebral disc (arrow).

Table 1. **Gross morphology criteria used to score segmental regions (AF and NP) shown in Figure 1 for each disc***

AF Morphological grades applied to each AF quadrant	NP Morphological grades applied to each half of NP.
Grade 0: **Normal Disc:** Normal disc, no annular disruption, discoloration or hemorrhage.	**Grade 0:** **Normal NP:** No discoloration or hemorrhage
Grade 1: **Minor Disruption:** Annular disruption with minor discoloration and/or hemorrhage	**Grade 1:** **Minor Disruption**: Minor disruption, discoloration and/or hemorrhage. <10% NP region. Minor fissuring and nuclear dehydration may be evident.
Grade 2: **Moderate Disruption:** Annular disruption with medium discoloration and/or hemorrhage.	**Grade 2:** **Moderate Disruption:** Medium disruption, discoloration and/or hemorrhage. 10-50% of NP region. Moderate fissuring and nuclear dehydration may be evident.
Grade 3: **Major Disruption**: Annular disruption with significant discoloration and/or hemorrhage.	**Grade 3:** **Major Disruption:** Significant disruption, discoloration and/or hemorrhage. 50-75% NP region. Major fissuring and nuclear dehydration may be evident.
Grade 4: Complete Disruption: Annular	**Grade 4:** **Complete Disruption:** Extensive

| disruption with extensive discoloration and/or hemorrhage. | disruption, discoloration and/or hemorrhage. >75% NP region. Extensive fissuring and dehydration may be evident. |

*The sum of all regional scores (AF1, AF2, AF3, AF4, NP1 and NP2) yielded a total disc degeneration score between 0 (normal) and 24 (severely degenerated) for each disc. (AF=Annnulus Fibrosis, NP=nucleus pulposus). Table is described in Daly et al.[26] and adapted from the method described by Oehme et al.[13].
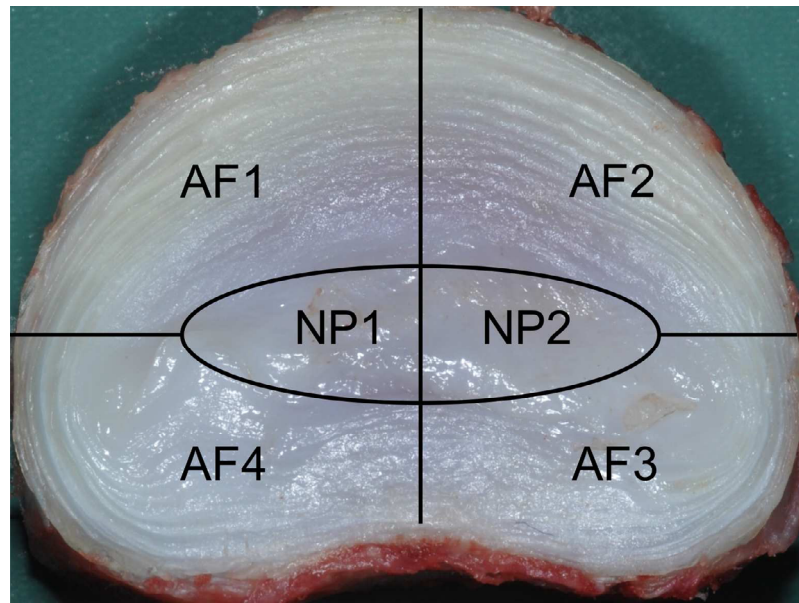
Fig. 1. Diagram showing the Intervertebral disc segments used for gross morphological and biochemical analysis. AF1 is the site of intervertebral disc annulotomy injury. NP1 is the region of NP on the injured half of the intervertebral disc. NP2 the complementary half of NP1. (AF= annulus fibrosus, NP= nucleus pulposus).

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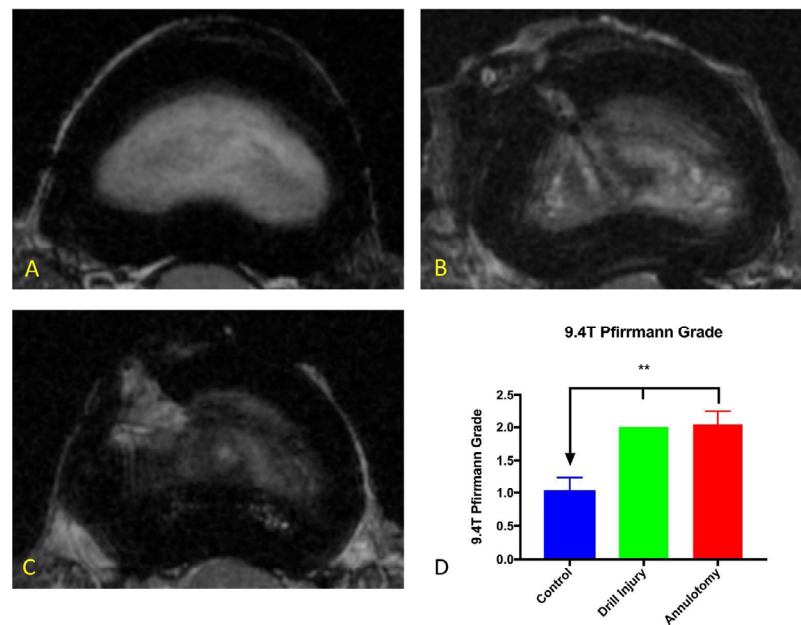
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Fig. 3A 9-4T MRI axial images A. Control disc demonstrating intact AF and hyerintense NP. B. Drill injury disc demonstrating injury tract (arrow) extending to NP C. Annulotomy disc demonstrating broader injury tract (arrow) and decreased NP hyperintensity. D. 9.4T MRI Pfirrmann grades Drill injury and annulotomy injured discs demonstrated significantly higher 9.4T Pfirrmann Grades than control discs with no signifcant difference between injured groups. (AF= annulus fibrosus, NP= nucleus pulposus) (* signifies p<0.05, ** signifies p<0.005).

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Fig. 4. Representative examples of disc gross morphology. A. Control disc demonstrating intact AF and gelatinous white NP (scored as 0). B. Drill injury disc demonstrating injury tract extending to NP (arrow) with discolouration of NP and AF disruption at injury site (scored as 4) C. Annulotomy disc demonstrating broader AF injury (arrow) with extension to NP, loss of NP material, discolouration of NP and AF (scored as 6). D. Gross morphology scores Drill injury and annulotomy injured discs demonstrated significantly higher gross morphology scores than control discs. Annulotomy discs demonstrated higher gross morphology scores than drill injury discs. (* signifies $p<0.05$, ** signifies $p<0.005$).

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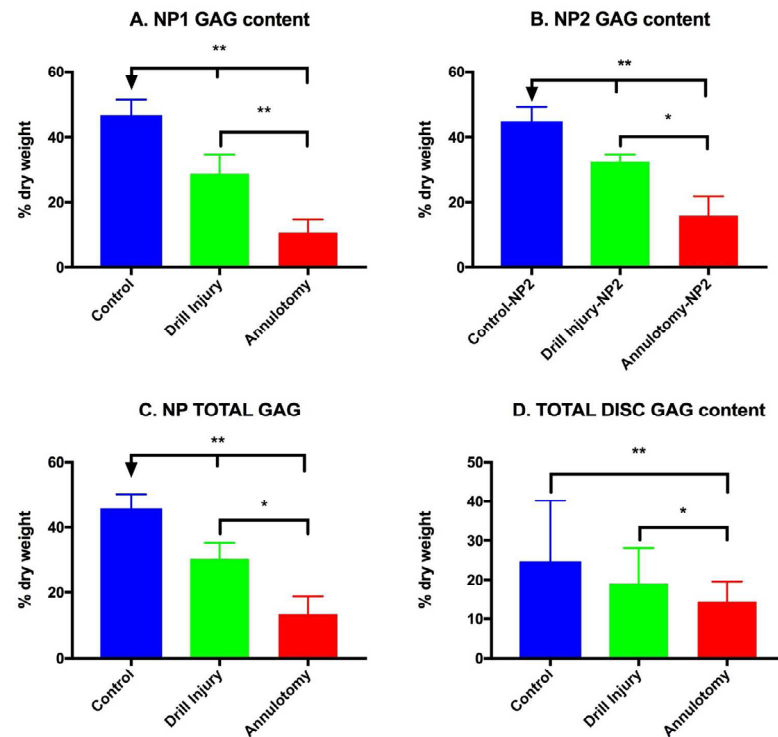
Fig. 5. Sulfated-GAG content (% dry weight). A. NP1 GAG is significantly lower in annulotomy than drill-injured discs which is in turn less than control discs. B. NP2 demonstrates the same relationship with annulotomy injured discs lower than drill injured discs (p<0.05) which in turn are less than control discs   C. NP TOTAL GAG and D. TOTAL DISC GAG demonstrates the same series of relationships. (* signifies p<0.05, ** signifies p<0.005).
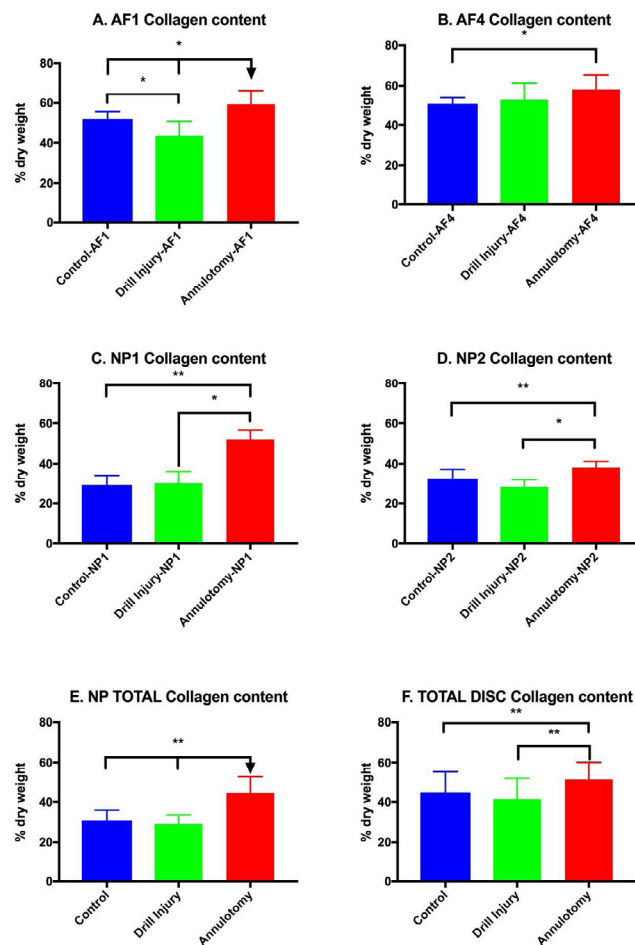
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Fig. 6. Collagen content (% dry weight). A. AF1 Annulotomy group discs had significantly more collagen than both control and drill injured discs. B. AF4 Annulotomy group collagen was significantly higher than control discs. C. NP-1 Annulotomy group collagen was significantly higher than both control and drill injury collagen. D. NP2 collagen demonstrated the same pattern. E. NP TOTAL also demonstrated an increase in collagen in the annulotomy group relative to the control group. F. TOTAL DISC collagen was significantly increased in the annulotomy group relative to both control and drill injured discs. (* signifies $p<0.05$, ** signifies $p<0.005$).
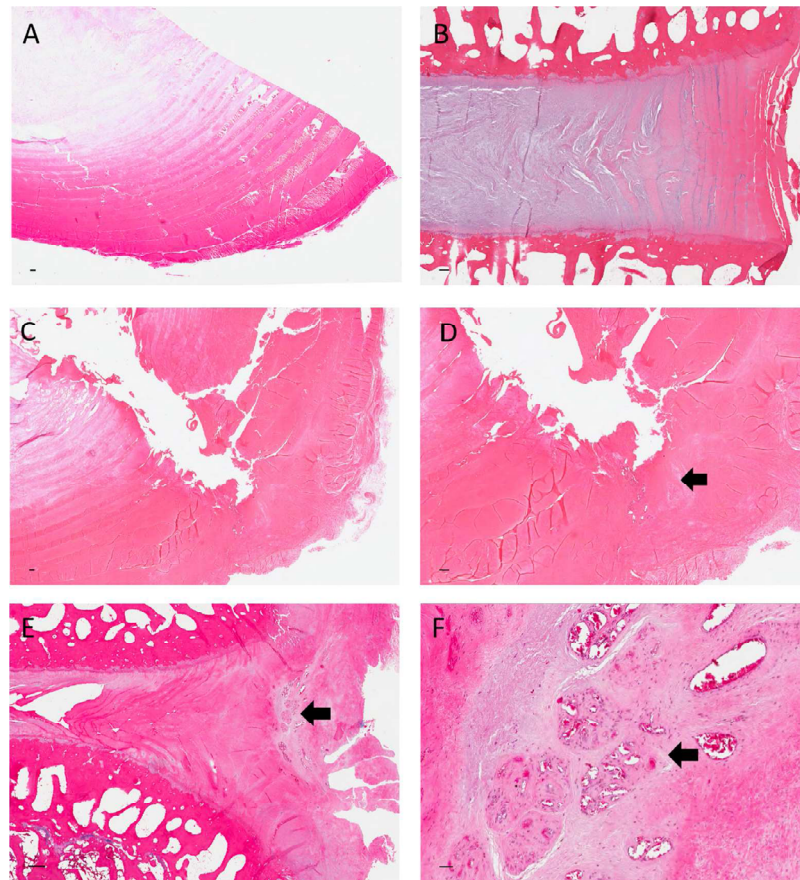
140x206mm (300 x 300 DPI)

Fig. 7. Sections stained with Hematoxylin & Eosin. A. Control disc (axial section) demonstrating intact annulus fibrosus and adjacent nucleus pulposus. B. Control disc (coronal section) demonstrating the coronal plane. C. Drill injured disc demonstrating disruption of the annulus fibrous extending into the nucleus. D. Drill injured disc under higher power demonstrating superficial localized fibrosis (arrow) at external AF with minimal vascular infiltration. E. Annulotomy injured disc demonstrating extensive lamellae disruption and vascular infiltration.   F. Annulotomy injured disc demonstrating lamellar structure under high power magnification with evidence of marked vascular invasion (arrow). Scale bar = 200 um.
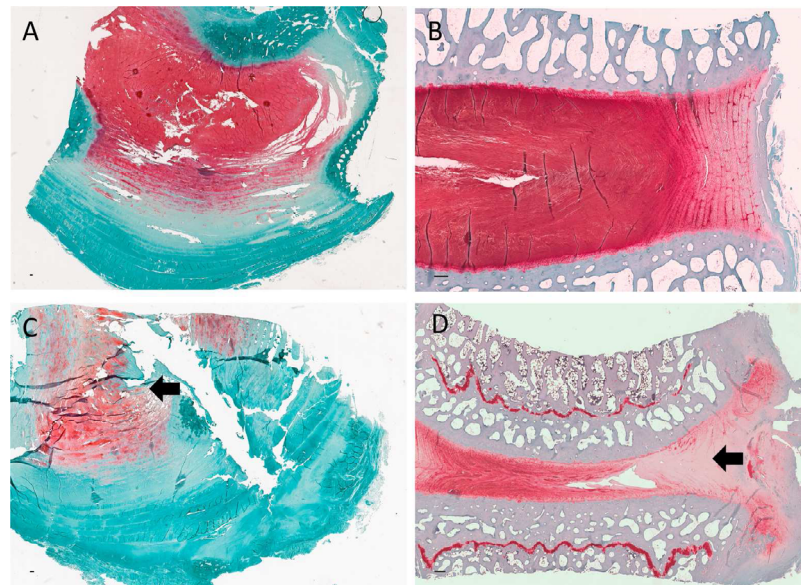
Fig. 8. Sections stained with Safranin O and Fast Green. A. Control disc (axial section) demonstrating intact annulus fibrosus and adjacent nucleus pulposus. B. Control disc (coronal section) demonstrating the coronal plane. C. Drill injured disc demonstrating reduced Safranin O stain (indicative of reduced proteoglycan content) in the drill injured region of the nucleus pulposus (arrow). D. Annulotomy injured disc demonstrating significant reduction in Safranin O stain in the injured region of the intervertebral disc (arrow).

173x125mm (300 x 300 DPI)

Chapter 4. Mesenchymal progenitor cells primed with pentosan polysulfate promote lumbar intervertebral disc regeneration in an ovine model of microdiscectomy

4.1. Introduction

This chapter contains a manuscript for an experimental study entitled, **"Mesenchymal progenitor cells primed with pentosan polysulfate promote lumbar intervertebral disc regeneration in an ovine model of microdiscectomy."** This manuscript has been published in The Spine Journal. This chapter describes a comparison of mesenchymal progenitor cells and pentosan polysulfate primed mesenchymal progenitor cells with regard to their ability to promote intervertebral disc regeneration in an ovine model of lumbar microdiscectomy.

The candidate, Chris Daly, contributed to the experimental design, experimental procedures and the writing of the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xviii.

Basic Science

Mesenchymal progenitor cells primed with pentosan polysulfate promote lumbar intervertebral disc regeneration in an ovine model of microdiscectomy

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Abstract

BACKGROUND CONTEXT: Neural compression associated with lumbar disc herniation is usually managed surgically by microdiscectomy. However, 10%–20% of patients re-present with debilitating back pain, and approximately 15% require further surgery.

PURPOSE: Using an ovine model of microdiscectomy, the present study investigated the relative potential of pentosan polysulfate-primed mesenchymal progenitor cells (pMPCs) or MPC alone implanted into the lesion site to facilitate disc recovery.

STUDY DESIGN: An ovine model of lumbar microdiscectomy was used to compare the relative outcomes of administering MPCs or pMPCs to the injury site postsurgery.

METHODS: At baseline 3T magnetic resonance imaging (MRI) of 18 adult ewes was undertaken followed by annular microdiscectomy at two lumbar disc levels. Sheep were randomized into three groups (n=6). The injured controls received no further treatment. Defects of the treated groups were implanted with a collagen sponge and MPC (5×10^5 cells) or pMPC (5×10^5 cells). After 6 months, 3T MRI and standard radiography were performed. Spinal columns were dissected, individual

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lumbar discs were sectioned horizontally, and nucleus pulposus (NP) and annulus fibrosus (AF) regions were assessed morphologically and histologically. The NP and AF tissues were dissected into six regions and analyzed biochemically for their proteoglycans (PGs), collagen, and DNA content.

RESULTS: Both the MPC- and pMPC-injected groups exhibited less reduction in disc height ($p<.05$) and lower Pfirrmann grades ($p≤.001$) compared with the untreated injury controls, but morphologic scores for the pMPC-injected discs were lower ($p<.05$). The PG content of the AF injury site region (AF1) of pMPC discs was higher than MPC and injury control AF1 ($p<.05$). At the AF1 and contralateral AF2 regions, the DNA content of pMPC discs was significantly lower than injured control discs and MPC-injected discs. Histologic and birefringent microscopy revealed increased structural organization and reduced degeneration in pMPC discs compared with MPC and the injured controls.

CONCLUSIONS: In an ovine model 6 months after administration of pMPCs to the injury site disc PG content and matrix organization were improved relative to controls, suggesting pMPCs' potential as a postsurgical adjunct for limiting progression of disc degeneration after microdiscectomy. © 2017 Elsevier Inc. All rights reserved.

Introduction

Low back pain is the leading cause of disability worldwide [1,2] and is strongly associated with disc degeneration [3,4]. Disc degeneration is a multifactorial disease with etiological contributions that include aging, physical activity, obesity, hormonal dysregulation, and genetics [5]. The degenerative process frequently extends to the annulus fibrosus (AF) and, when coupled with physical activity, may lead to annular failure, disc herniation, and subsequent compression of the neural elements producing radicular pain or radiculopathy. When symptomatic herniation with neural compression fails conservative management, it may be treated surgically with lumbar microdiscectomy.

Lumbar microdiscectomy is the most common spinal operation performed globally with over 300,000 such procedures undertaken annually in the United States alone [6]. Unfortunately, lumbar microdiscectomy, although relieving the radicular symptoms, fails to address the underlying pathophysiology of disc degeneration that contributed to the annular failure. Consequently, many patients continue to experience back pain that may be disabling in up to 10% of those who have undergone microdiscectomy [5]. Recurrent disc herniation occurs in 5%–15% of patients and reoperation may be required in 4%–25% of this cohort [7]. A recent retrospective analysis of US national insurance databases revealed that 12% of patients subjected to microdiscectomy will undergo reoperation for the same procedure within 4 years [7]. Approximately 40% of these patients will subsequently progress to fusion surgery within 4 years [7].

Given the global disease burden of back pain and the failure of conventional therapies to address the underlying pathobiology of disc degeneration, concerted efforts to develop alternative modalities of treatment are currently under investigation. Mesenchymal stem cells (MSCs) have long been used in spinal surgery, originally in the form of the iliac bone marrow explants, to promote bone fusion, but more recently in their purified form where they have been evaluated as potential candidates to support disc repair [8–18]. Although these earlier studies were undertaken using animal models of experimentally induced disc degeneration, several human clinical trials, using both autologous and allogeneic MSCs, have been reported [19]. In general, these clinical studies have demonstrated relief of symptoms in patients with discogenic back pain, improved outcome measures, and some have further shown improved intervertebral disc water content on magnetic resonance imaging (MRI) or reduced spinal translational movement [20–25].

Mesenchymal stem cells can be derived from a variety of tissues. However, the most frequently used source is from the stromal tissue present within the perivascular niche of the bone marrow. Aspirates obtained from bone marrow consist of a heterogeneous population of cells from which MSCs may be isolated by centrifugation, followed by their adherence to plastic flasks and subsequent in vitro culture expansion [26]. MSCs prepared from adult human bone marrow by these means contain a low abundance of highly proliferative, immature mesenchymal progenitor cells (MPCs) capable of extensive population doublings and are contaminated by low proliferative colony-forming MSCs and non-mesenchymal cell types [27,28]. Notably, studies show that MPCs are the major colony-forming population present within the bone marrow and exhibit higher plasticity than the mature stromal cells [28–30]. MPCs can be readily isolated from the bone marrow aspirates using the technique of magnetic-activated cell-sorting-based immunoselection using monoclonal antibodies that selectively bind to specific antigens expressed on the surface of the MPCs [29,30]. Examples of these antibodies include stromal cell surface marker-1 (STRO-1), STRO-3, STRO-4, heat shock protein-90b, vascular cell adhesion molecule-1, cluster of differentiation 106 (CD106), and CD146 [28–30].

A previous study by our group used immune-selected STRO-3+MPCs, prepared from ovine bone marrow aspirates, to investigate the reconstitution of lumbar discs in an

ovine model of disc degeneration, mediated by injection of the enzyme chondroitinase-ABC into the nucleus pulposus (NP) [9]. Although this study demonstrated that MPCs were effective in supporting recovery of disc proteoglycans (PGs) and disc height index (DHI), this animal model failed to replicate the hallmark pathologic features of human disc degeneration, namely, loss of cell viability and the absence of concentric tears within the AF, accompanied by vascular invasion [31–33]. In addition, subsequent in vitro [34] and in vivo [17] studies demonstrated that combination of MPC with the pharmaceutical agent, pentosan polysulfate (PPS), not only enhanced their proliferation and chondrogenic differentiation but also suppressed osteogenesis. Using an ovine model of lumbar disc degeneration, induced by the surgical creation of a horizontal lesion in the periphery of their AFs, it was reported [10] that 6 months after the injection of a formulation of MPCs with PPS into the NP of degenerate discs, increased disc height, reduced morphologic evidence of matrix failure, and significantly elevated PG content of their NP relative to controls were observed. The same ovine formulation of MPCs and PPS used in the ovine disc degeneration model [10] was also evaluated in an ovine model of microdiscectomy [8]. In this pilot study [8], a suspension of allogeneic MPCs with PPS was applied to a gelatin sponge scaffold placed in a full depth defect created in the AF of ovine lumbar discs then closed with fibrin glue. The two adjacent lumbar discs were used as untreated controls. Following euthanasia 6 months later, it was observed that the microdiscectomy defects implanted with the MPC+PPS and gelatin sponge exhibited significantly more new matrix regeneration and preservation of disc height compared with defects that were untreated or received the scaffold alone [8].

Although the pilot study provided important information in regard to a potential surgical adjunct to support disc repair following microdiscectomy, since PPS was known to promote regeneration of cartilage in osteoarthritic joints [35], it was unclear whether the MPC or PPS were the active mediators of the repair observed in this model or whether both components acted synergistically. To resolve this question, an in vitro study was undertaken to identify the mechanism of action of combining PPS with MPCs [36]. The results of this study indicated that the activation of MPC by PPS in culture was mediated by its migration to nucleus of the MPCs, where it upregulated specific gene pathways responsible for cell replication, differentiation, and biosynthesis of PGs [36]. Significantly, these studies also demonstrated that after culturing the MPCs with PPS for 24–48 hours, then eliminating the presence of PPS from the cultures, the MPCs retained their genetic re-programing, that is, they were now "primed" to differentiate to a chondrogenic phenotype, and as such would be superior to MPCs alone in supporting repair of injured cartilaginous tissues [36].

The objective of the present study was to test the hypothesis that PPS-primed MPCs (pMPCs) administered into a disc defect created by microdiscectomy were capable of facilitating repair.

Materials and methods

Surgical procedure

With ethics approval from the Monash Medical Centre Animal Ethics Committee and conforming to the Australian code of practice for the care and use of animals for scientific purposes (8th edition, 2013), 18 adult Border-Leicester Merino cross-bred ewes underwent preoperative 3 T MRI (Siemens Skyra Widebore 3T MRI, Siemens, Erlangen, Germany) under general anesthetic. All sheep were open pasture raised and freely ambulating before the trial. Sheep were fasted for 24 hours before surgery and anesthetized using intravenous thiopentone (10–15 mg/kg) (Bayer Australia Ltd, Pymble, NSW, Australia) followed by intubation and isoflurane inhalation (Pharmachem, Eagle Farm, QLD, Australia) (2%–3% in oxygen). Sheep were placed in the right lateral position. Following the subcutaneous administration of local anesthetic (bupivacaine 0.5%) (AstraZeneca Australia, Macquarie Park, NSW, Australia), the L2–L3 and L3–L4 lumbar intervertebral discs were exposed via left lateral retroperitoneal approach, as previously described [37,38]. Intraoperative lateral radiographs (Radlink, Atomscope HF200A, Redondo Beach, CA, USA) were performed to confirm the correct levels. The microdiscectomy annulotomy injury was performed by creating a 3-mm×5-mm window incision in the annulus using a pituitary rongeur. The disc tissues collected (200.0±3.0 mg) consisted mainly of AF and the outer region of the NP. The adjacent L1–L2 and L4–L5 discs served as untreated controls.

Following the microdiscectomy procedure, sheep were randomized to one of the three treatment groups (six sheep per group). The injury control group received no further therapy and served to replicate current clinical practice. The MPC-alone group received 0.5×10^6 MPCs delivered onto a 5×5×3-mm gelatin sponge (Gelfoam, Pfizer Ltd, West Ryde, NSW, Australia) placed within the defect followed by closure of the outer region with a fibrin sealant (Tisseel, Baxter International Inc, Old Toongabbie, NSW, Australia) to prevent any efflux of the cell suspension out of the defect.

The PPS-primed MPC (pMPC) group received 0.5×10^6 pMPC applied to the gelatin sponge and closed with fibrin sealant by the same procedure as described for the MPC-alone group.

After gelation of the fibrin, the wound was closed using a routine layered procedure performed using absorbable sutures (Vicryl, Ethicon, NJ, USA). Animals received a fentanyl patch (Duragesic 75 μg/h, Jannsen LLC, North Ryde, NSW, Australia) and intravenous paracetamol (Pfizer Ltd, West Ryde, NSW, Australia) for postoperative analgesia. Following recovery, animals were returned to the pen with other sheep and allowed free ambulation.

Sheep were transferred to open pasture 1 week postsurgery. Necropsy was performed at 6 months.

Primed and non-primed MPCs

A stock batch of the ovine MPCs used for these studies were prepared from iliac crest bone marrow aspirates of adult Border Leicester Merino crossbred sheep screened for mycoplasma and other ovine pathogens using our published procedures [30]. The protocol used for PPS priming of MPCs derived from the MPC stock batch cultures has been described previously [36]. The PPS used was commercially available pharmaceutical grade PPS provided by BenePharmachem GmbH & Co KG, Munich, Germany. The phenotype of the MPCs and pMPC used for the study was confirmed using multilineage differentiation assays and flow cytometry that demonstrated that both MPCs and pMPCs expressed the characteristic MSC markers CD73, CD90, CD105, CD44, and CD 146, but exhibited low expression of the hemopoietic and vascular endothelial markers: CD14, CD34, and CD45 [29]. MPCs were stored in liquid nitrogen and transferred to the operating theater frozen then defrosted using the standard procedures immediately before use. Before administration, cell numbers and viability were determined using a Neubauer hemocytometer (Invitrogen, Carlsbard, CA, USA) as previously described [8]. Where cell counts or viability were less than 80%, cells were disposed of and a new vial used.

Necropsy

At 6 months following surgery, all animals were euthanized by intravenous injection of 150 mg/kg of pentobarbital (Sigma-Aldrich, Castle Hill, NSW, Australia). Lateral lumbar spinal digital radiographs of all sheep were obtained with the lumbar spine in situ. The lumbar spines were then removed en bloc, a segment was isolated from the mid-sacrum to the thoracolumbar junction, placed on ice, and transferred to Monash Biomedical Imaging for MRI analysis (Siemens Skyra Widebore 3T MRI). All spines were subjected to radiological analysis using radiography and MRI. Spinal columns were then transected in the horizontal plane through their vertebral bodies using a band saw to afford spinal segments that included complete lumbar discs with half of the adjacent vertebral bodies attached. Subsequent gross morphologic, biochemical, and histologic analysis of discs were undertaken using these spinal segments as described below. Spinal segments containing discs destined for histologic analysis were transferred to phosphate buffered formalin.

Radiological analysis

Using standardized methods, DHI measurements were calculated and recorded by an observer blinded to the treatment regimen, using standard digital processing software (Osiris MD v8.0.2, Pixmeo, Geneva, Switzerland).

Sagittal 3T T2-weighted MRI sequences of the entire lumbar spine explant were obtained for each animal. Using sagittal T2-weighted sequences, four blinded observers (a neuroradiologist, a neurosurgeon, and two neurosurgery
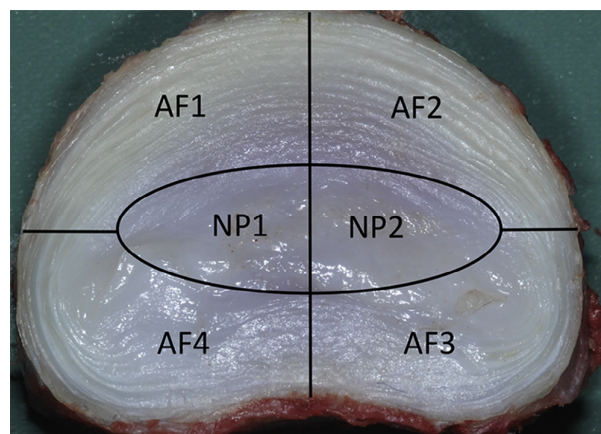

Fig. 1. Diagram showing the intervertebral disc segments used for gross morphologic and biochemical analysis. AF1 is the site of intervertebral disc annulotomy injury. NP1 is the region of NP on the injured half of the intervertebral disc. NP2 is the complementary half of NP1. AF, annulus fibrosus; NP, nucleus pulposus.

residents blinded to the treatment regimen) determined the Pfirrmann MRI disc degeneration scores for all lumbar discs.

Disc height index analysis was also performed using the preoperative and 3T MRI images determined at necropsy by an observer blinded to the intervention protocol. The 3T MRI assessment of the DHI eliminated the potential for parallax error while also producing consistent image quality for all discs.

Gross morphologic analysis

Lumbar spinal disc segments allocated for gross morphologic and biochemical analysis were sectioned in the horizontal (axial) plane using a 100.0×25.0×2.5-mm blade to provide two complementary halves of the disc as shown diagrammatically in Fig. 1. High-resolution digital photographs were taken of the exposed complementary surfaces and each region shown in Fig. 1 scored by two blinded observers (CD, PG) using the scoring system shown in Table that was adapted from the method described by Oehme et al. [10].

Biochemical analysis

Following collection of the digital images of discs from each lumbar spinal level for morphologic analysis, all tissue regions shown in Fig. 1 were subjected to biochemical analysis. The individual NP and AF were separated from each other and their vertebral attachments by careful dissection following the boundaries shown in Fig. 1. Tissues from each region were finely diced, frozen in liquid N_2, and powdered in a liquid Nitrogen cooled ball-mill. The powdered tissues were transferred to pre-weighed Eppendorf vials and weighed, lyophilized, and then reweighed to constant weight to determine their anhydrous weights. Aliquots (3×) of the dehydrated tissues were subsequently solubilized using a papain digestion

Table
Gross morphology criteria used to score AF and NP segmental regions shown in Fig. 1 for each disc*

AF Morphologic grades applied to each AF quadrant (AF1, AF2, AF3, and AF4)	NP Morphologic grades applied to each half (NP1 and NP2) of NP.
Grade 0: **Normal disc:** Normal disc, no annular disruption, discoloration, or hemorrhage.	**Grade 0: Normal NP:** No discoloration or hemorrhage.
Grade 1: Minor disruption: Annular disruption with minor discoloration or hemorrhage.	**Grade 1: Minor disruption**: Minor disruption, discoloration, or hemorrhage. <10% NP region. Minor fissuring and nuclear dehydration may be evident.
Grade 2: Moderate disruption: Annular disruption with medium discoloration or hemorrhage.	**Grade 2: Moderate disruption:** Medium disruption, discoloration, or hemorrhage. 10%–50% of NP region. Moderate fissuring and nuclear dehydration may be evident.
Grade 3: Major disruption: Annular disruption with significant discoloration or hemorrhage.	**Grade 3: Major disruption:** Significant disruption, discoloration, or hemorrhage. 50%–75% NP region. Major fissuring and nuclear dehydration may be evident.
Grade 4: Complete disruption: Annular disruption with extensive discoloration or hemorrhage.	**Grade 4: Complete disruption:** Extensive disruption, discoloration, or hemorrhage. >75% NP region. Extensive fissuring and dehydration may be evident.

AF, annulus fibrosus; NP, nucleus pulposus.
Table is adapted from the method described by Oehme et al. [10].
* The sum of all regional scores (AF1, AF2, AF3, AF4, NP1, and NP2) yielded a total disc degeneration score between 0 (normal) and 24 (severely degenerated) for each disc.

buffer (50 mM sodium acetate {pH=6.0]) containing 2 mg/mL papain (Sigma-Aldrich Chemicals, Sydney, NSW, Australia) by incubation at 60°C for 16 hours [39]. The digested tissues were then centrifuged at 3000g for 15 minutes and supernatants diluted to a standard volume (the stock digest solution). Aliquots of the stock solution were analyzed for sulfated glycosaminoglycan content (S-GAG) (an index of PG content) using the dimethylmethylene blue assay [40], hydroxyproline assay (to derive collagen content) [41], and Hoechst dye 33258 assay for DNA content [42] as an index of cell numbers. The results of biochemical analyses are expressed as percentage of tissue dry weight for S-GAG, collagen, and DNA.

Histologic analysis

The individual disc segments, consisting of the intervertebral disc with the sawn vertebral bodies attached were in 10% neutral buffered formalin for 8 days then transferred to 70% ethanol for storage. The volume of vertebral bone was reduced down to the growth plate using a fine diamond saw. Before paraffin based-tissue embedding, decalcification of the remaining vertebral bone was undertaken with multiple changes of 10% formic acid. Coronal paraffin sections of the entire disc segments were cut using a standard rotary microtome and stained using hematoxylin and eosin (H&E), and Alcian blue or Picrosirius red employing standard protocols. However, because of the extended time required to completely decalcify the vertebral bone, coupled with marked disruption of the AF tissue integrity in the surgical zone, the quality of the disc tissue sections cut from the untreated microdiscectomy sections were considered unacceptable for quantitative histologic scoring. Nevertheless, all histologic sections were reviewed qualitatively. In addition, the H&E sections were examined by polarized light microscopy to identify changes of AF collagen fiber orientation using birefringence.

Images were captured using a charge-coupled device camera mounted on a Leica DMIRB base microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany). Images were analyzed using Abrio software (CRI, Woburn, MA, USA) that allowed additional qualitative assessments between the experimental groups to be determined.

Statistical analysis

All data analysis and storage was performed using Prism 7.0c for Mac (GraphPad Software Inc, La Jolla, CA, USA) and Microsoft Excel for Mac (Version 15.33, Microsoft, Redmond, WA, USA). Parametric data were analyzed using one-way analysis of variance, and the Tukey multiple comparison test was performed when significant differences in means were observed. Non-parametric data were analyzed using Kruskal-Wallis test of median values followed by Dunn multiple comparison test. Treated groups were compared using the two-tailed Student *t* test followed by Mann-Whitney *U* test. A p-value <.05 was considered statistically significant.

Results

Disc height

Using only the MRIs, it was demonstrated that the pMPC- and MPC-treated discs' loss of DHIs were significantly less than the injury control discs (p<.05) (Fig. 2, Left). There was no significant difference between pMPC- and MPC-treated discs. However, the percentage loss of DHI observed between all microdiscectomy-treated levels (pMPC, MPC, and injury) and normal controls was significant (p<.05 for all groups).

Pfirrmann grades

Baseline preoperative MRIs revealed no evidence of underlying disc degeneration. However, 3T MRI radiological
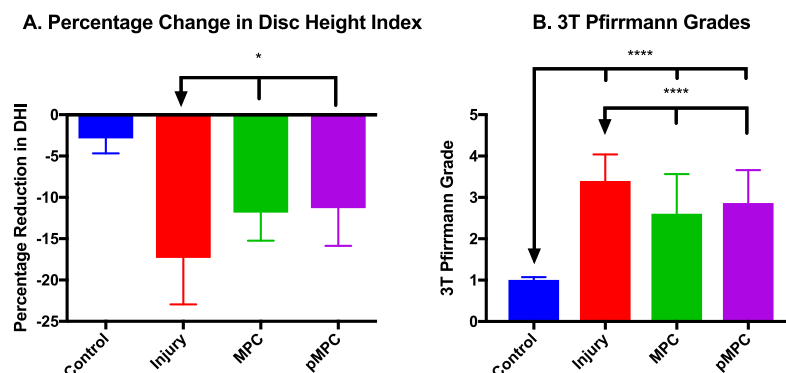
Fig. 2. (Left) Percentage change in disc height index. The MPC- and pMPC-treated group demonstrated significantly less loss of DHI relative to non-treated injury discs. However, the percentage decline in the control group was significantly less than all injured and treated groups (p<.05). (Right) 3T Pfirrmann grades. MPC and pMPC Pfirrmann grades were significantly reduced relative to injured discs. All intervention groups had significantly increased Pfirrmann grades relative to the control group. DHI, disc height index; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell. * p<.05; ** p≤.01; *** p≤.005; **** p≤.001.

analysis demonstrated a significant reduction in Pfirrmann grade for the MPC-treated (p<.001) and pMPC-treated (p=.001) disc, relative to injured untreated discs (Fig. 2, Right). As observed with DHI analysis, there was no significant difference in Pfirrmann grade between the MPC- and pMPC-treated discs (Fig. 2, Right). The Pfirrmann grade for control discs was significantly lower than injured and treated discs.

Gross morphologic analysis

Representative gross morphologic images and their scores, determined using the scheme described in Table, for each experimental group are shown in Fig. 3. Total disc morphology scores for the pMPC-treated group were significantly less than injured controls (p<.05) (Fig. 3E). Combined NP gross morphology scores for pMPC-treated discs, for regions NP1+NP2, were significantly lower than injured discs (p<.05) but not significantly different from normal controls (Fig. 3F).

Proteoglycan content as determined by sulfated-glycosaminoglycans (S-GAG) analysis

Analysis of all AF injury sites (AF1), irrespective of treatment, showed that the S-GAG content was reduced relative to the corresponding region of the uninjured control discs. However, the AF injury site (AF1) of pMPC-treated discs exhibited significantly higher S-GAG levels, relative to both injured (p<.05) and MPC-treated discs (p<.05) (Fig. 4A). In the AF region, immediately adjacent to the injury site (AF4), injured discs (p<.05) and pMPC-treated discs (p<.05) had significantly higher S-GAG compared with MPC-treated discs (Fig. 4B). Moreover, the S-GAG levels in the pMPC-treated discs were not significantly different from controls (Fig. 4B). No significant differences were observed between groups in the AF2 and AF3 regions (data not shown).

Determination of the S-GAG content of the tissue from the NP1 region of injured discs (Fig. 4C) showed that the pMPC-treated discs contained higher S-GAG levels than either

the injury or MPC-treated discs; however, this difference did not achieve statistical significance. All injured and treated discs had significantly lower NP1 S-GAG content than controls. The NP region, contralateral to the site of injury (NP2), demonstrated no significant difference in S-GAG content between untreated control and pMPC-treated discs and higher levels than the corresponding injured disc NP2 region (p<.05) but was not significantly different from MPC-treated discs (Fig. 4D). Assessment of the combined S-GAG levels of the NP1 and NP2 regions (total NP) revealed that the MPC- and pMPC-treated discs had significantly higher total NP S-GAG levels than injured discs (p<.05) but were not significantly different from each other (Fig. 4E). Analysis of a total of S-GAG levels for all disc regions showed that pMPC-treated discs had significantly higher S-GAG content than injured discs (p<.05).

Collagen content of disc regions

Biochemical analysis of the four AF regions revealed that only the injury site AF (AF1) and AF4 regions showed significant changes in collagen content relative to controls. For the AF1 region of the pMPC-treated discs, the collagen content was not significantly different from control discs. However, the levels of this protein in the injury and MPC-treated discs were higher than controls (Fig. 5A, B). In contrast for all injured discs, treated or untreated, the collagen content of the two NP regions were all higher than the corresponding uninjured controls (Fig. 5C, D).

DNA

The DNA content of the AF1 regions was significantly higher in injured untreated discs than in pMPC-treated discs (p<.005) and control discs (p<.005) although not significantly different from MPC-treated discs (Fig. 6A). In addition, the DNA content of the region adjacent to the injury site (AF2) was also found to be significantly less in pMPC-treated discs
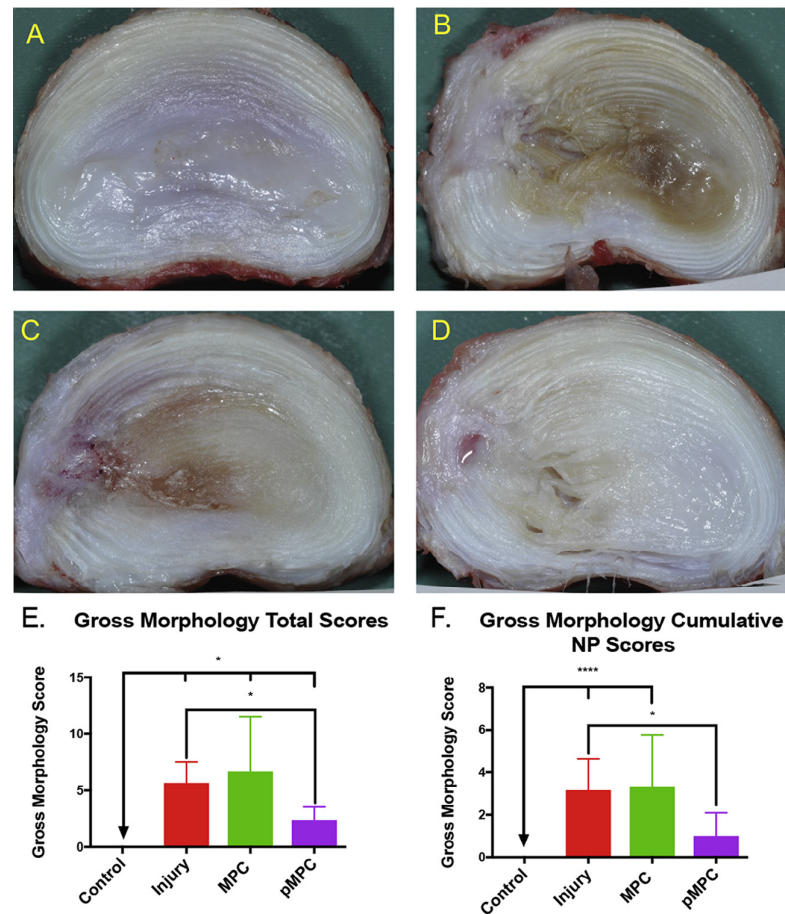
Fig. 3. Representative examples of disc gross morphology scoring. (A) Control uninjured AF and NP (scored as 0). (B) Injured disc disruption of AF with discoloration and disruption of ipsilateral and contralateral NPs (scored as 7). (C) MPC reduced disruption of the NP relative to the injured disc is evident (scored as 5). (D) pMPC relative preservation of NP structure and coloration evident. Minimal disruption to ipsilateral NP or AF evident (scored as 2). (E) Total disc gross morphology scores. All injured and treated groups are significantly different from the control group. (F) Cumulative NP gross morphology scores. The pMPC-treated discs have significantly lower gross morphology scores than untreated injury discs and are not significantly different from the control discs (n=6 discs per group). AF, annulus fibrosus; NP, nucleus pulposus; MPC, mesenchymal progenitor cell, pMPC, pentosan polysulfate-primed mesenchymal progenitor cell. * $p<.05$; ** $p\leq.01$; *** $p\leq.005$; **** $p\leq.001$.

than injured discs ($p<.05$), but not significantly different from control discs (Fig. 6B). Surprisingly, in this AF region (AF2), the mean DNA content of the MPC-treated discs was found to be lower than both pMPC-treated and untreated injured discs ($p<.05$) (Fig. 6B).

The NP DNA content at the site of injury (NP1) was significantly less in the pMPC-treated discs relative to the injured discs ($p<.05$), whereas the level in the MPC-treated group was equivalent to that of the untreated injury disc region (Fig. 6C). Significantly lower DNA levels were also found in the contralateral NP (NP2) region of the pMPC discs compared with the untreated injured control discs ($p<.005$) and were equivalent to control discs. MPC-treated discs also contained less DNA content than injured discs ($p<.05$) (Fig. 6D). However, the total DNA content of the NP of the pMPC- and MPC-treated discs were similar and both were not significantly different from control discs (Fig. 6E). Notably, only the

pMPC-treated discs exhibited significantly lower DNA content than injured discs ($p<.005$) (Fig. 6E). Analysis of the total DNA content of the AF regions of pMPC- and MPC-treated discs both revealed significantly lower levels than the injured disc controls ($p<.001$) (Fig. 6F).

Histology

Qualitative analysis of the histologic sections revealed differences among the interventions with respect to their vascular infiltration, PG content, and disc ultrastructure. The normal control sections stained with H&E demonstrate clearly the lamellation of the intact AF and the central convex NP (Fig. 7A). Sections from normal controls stained with Alcian blue and counterstained with Picrosirius red (Fig. 8A) identified the high content of PGs in the disc NP with lesser staining for PGs in the inner AF region. The
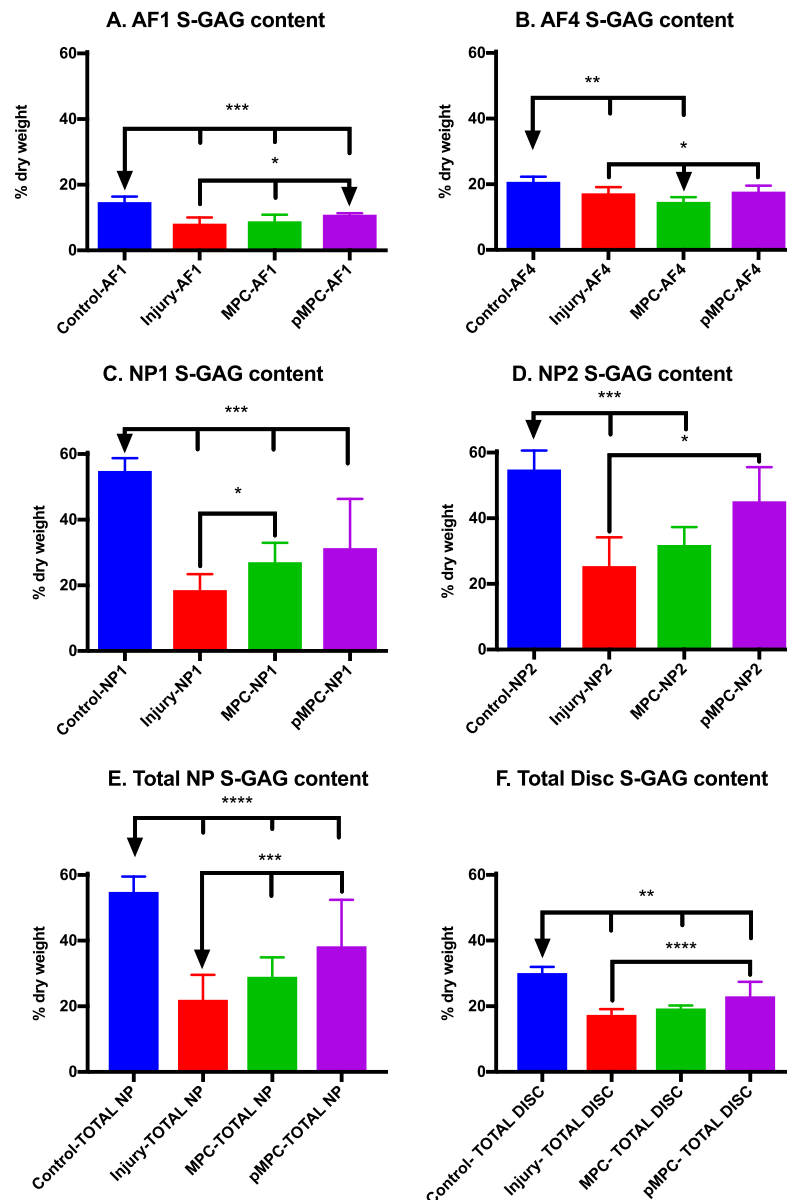
Fig. 4. Sulfated-GAG content (% dry weight). (A) AF1 injured region of pMPC group demonstrates higher S-GAG levels than injury and MPC groups. All injured and treated groups have significantly less GAG than control discs. (B) AF4 pMPC-treated discs have significantly higher GAG than MPC discs and are not significantly different from control. Injured and MPC discs have significantly lower S-GAG levels than control discs. (C) NP1 MPC group demonstrates significantly higher GAG levels than injury groups. (D) NP2 pMPC discs have higher S-GAG than injured discs and are not significantly different from control discs. (E) Total NP MPC and pMPC discs had higher S-GAG than injured discs. (F) Total disc pMPC-treated discs demonstrated significantly increased GAG compared with injury discs. GAG, glycosaminoglycans; S-GAG, sulfated glycosaminoglycans; AF, annulus fibrosus; NP, nucleus pulposus; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell. * p<.05; ** p≤ign; *** p≤igni; **** p≤igni.

organized collagen distribution of the lamellae of the peripheral AF and subchondral bone was highlighted by the intensity of the brown staining with the Picrosirius red dye (Fig. 8A). The loss of Alcian blue staining for the NP and inner AF regions in the section from the non-treated injured disc was consistent with the loss of PGs from these regions (Fig. 8B). Vascular infiltration at the injury site was a common feature of injured discs but was most evident in the MPC-treated groups, where the capillary infiltration into the normally avascular inner AF and NP interface were readily identified (Fig. 8D) as shown in detail in the H&E stain (Fig. 7D).

A notable finding was the minimal capillary invasion in sections from the pMPC-treated group (Figs. 7E, F and 8E, F), relative to the injury and MPC-treated discs (Figs. 7C, D and 8C, D). Relative preservation of the AF lamellae
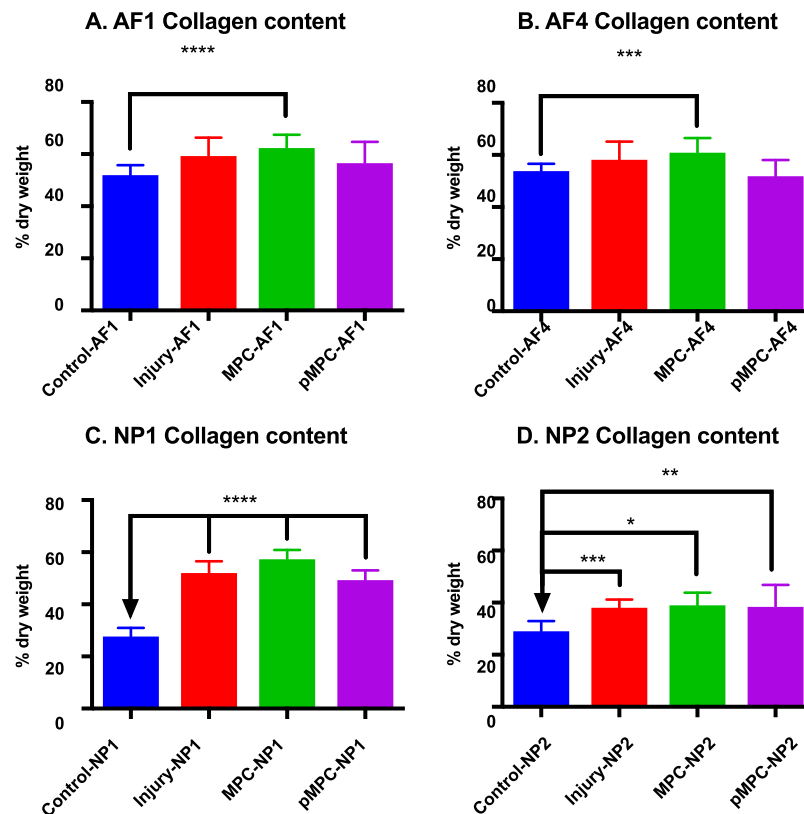
Fig. 5. Collagen content (% dry weight). (A) AF1 MPC collagen content was significantly increased relative to control. (B) AF4 MPC was significantly higher than control. (C) NP1 All injury and treatment groups were significantly higher than control. (D) NP2 All injury and treatment groups were significantly higher than control. AF, annulus fibrosus; NP, nucleus pulposus; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell. * $p<.05$; ** $p\leq.01$; *** $p\leq.005$; **** $p\leq.001$.

structure could also be observed in the pMPC groups sections (using the Alcian blue or Picrosirius red stains; Fig. 8E, F).

The structural integrity of the AF lamellae of the discs was also investigated using birefringent light microscopy (Fig. 9). In uninjured control discs (Fig. 9A), the unique transition of the collagen lamellae sheets extended from the caudal and cranial vertebral attachments through the cartilaginous end plates. The random arrangement of the collagen fibers in the NP did not elicit birefringence of the polarized light and were only visualized as a dark field image (Fig. 9A). As is evident in Fig. 9B, microdiscectomy provoked massive disruption of the AF lamellae with the severed ends interrupted by dark space. At the periphery of the AF, early repair was evident by the random deposition of collagen, surrounded by non-birefringent connective tissues and blood vessels. In the MPC- and pMPC-treated groups, the birefringent images revealed the presence of more abundant collagen fiber organization (Fig. 9C, D). However, for the injured discs treated with the pMPCs, these collagen fibers were more ordered and showed evidence of contiguous connections between the vertebral bodies within the inner and central AF regions (Fig. 9C).

Discussion

The present studies demonstrated that both MPCs and PPS-primed MPCs, when deposited onto a degradable collagen sponge and implanted into a full annular defect then sealed with fibrin, promoted disc repair to a greater extent than non-treated injured control discs. However, biochemical analysis revealed that the MPCs primed with PPS achieved deposition of higher levels of PG within injured discs than MPCs alone. Moreover, the biochemical data were consistent with the observations of improved matrix integrity, as identified by histologic and birefringent microscopy and lower gross morphologic scores. These data are supportive of our hypothesis that short-term exposure, or "priming" of MPCs with PPS, is capable of genetically re-programming MPCs toward the chondrogenic lineage, thereby rendering these cells more effective in supporting repair of injured disc tissues such as occurs following microdiscectomy without the likelihood of osteogenesis and potential spinal fusion [43].

Loss of PGs and their associated water from the disc is an early event in the pathophysiology of disc degeneration [44] and forms the basis for detecting degenerative changes by MRI and application of the Pfirrmann scoring system [45].
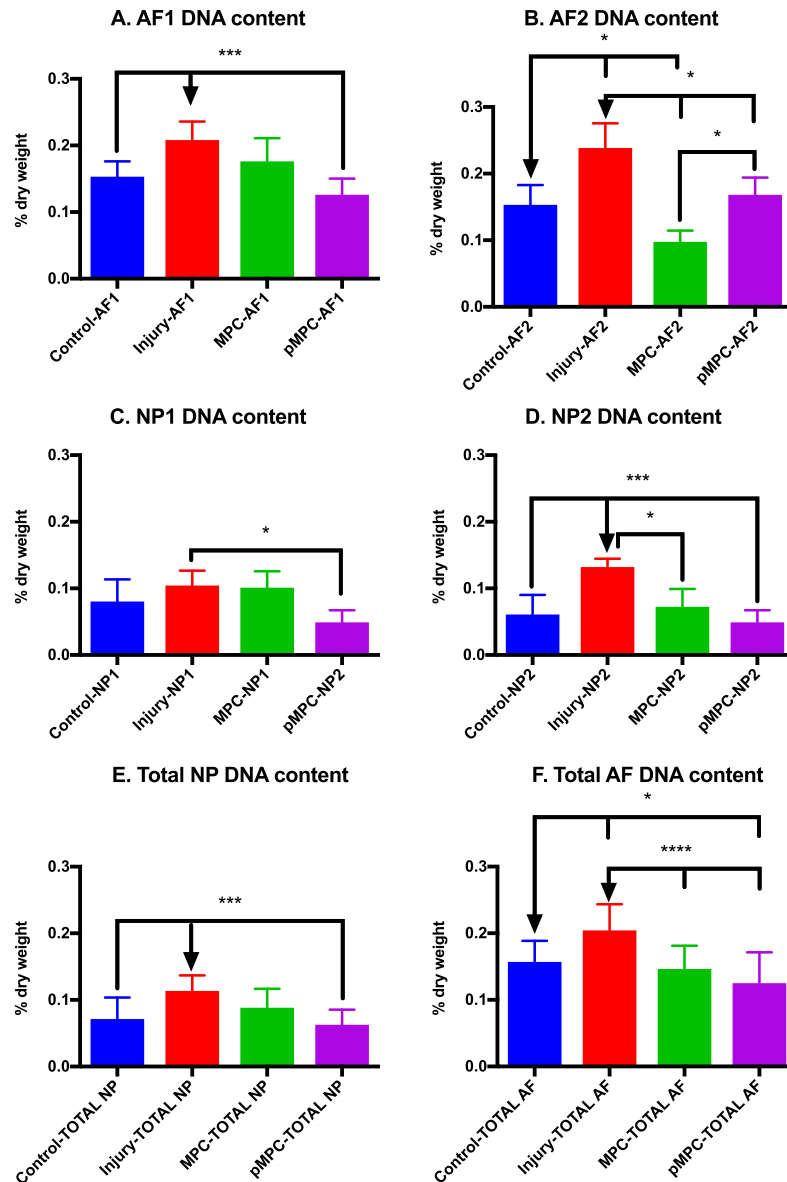
Fig. 6. DNA content (% dry weight). (A) AF1 pMPC-treated discs demonstrated significantly reduced DNA content compared with injured discs. Both MPC- and pMPC-treated discs were not significantly different from control disc. (B) AF2 pMPC-treated discs are not significantly different from control discs and demonstrate decreased DNA relative to injured discs. MPC DNA is significantly lower than control, injured discs, and pMPC discs. (C) NP1 pMPC discs demonstrate significantly lower NP1 DNA than injured discs. (D) NP2 pMPC and MPC discs had significantly lower NP1 DNA than injured discs and were not significantly different from control discs. (E) Total NP pMPC-treated discs have significantly lower NP-combined DNA content than injured discs. MPC- and pMPC-treated discs are not significantly different from control discs. (F) Total AF pMPC- and MPC-treated discs demonstrate significantly reduced AF total DNA relative to injured discs. MPC discs were not significantly different from control discs. AF, annulus fibrosus; NP, nucleus pulposus; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell. * p<.05; ** p≤.01; *** p≤.005; **** p≤.001.

In the present study, the Pfirrmann scores and DHI changes for the pMPC- and MPC-treated groups were found to be equivalent but inconsistent with the biochemical findings which showed that the level of PGs in the pMPC group were higher than observed for the MPC group. We suggest that this discrepancy between the MRI and biochemical findings could be attributed to the relatively low sensitivity of conventional

MRI as a means of assessing early disc degeneration and DHI. Moreover, the method for the determination of PG content is objective, whereas the Pfirrmann scoring system depends on subjective assessment.

Furthermore, as already discussed, disparity of the biochemical results was not observed for the other parameters examined in this study. Thus, the PG content of discs was
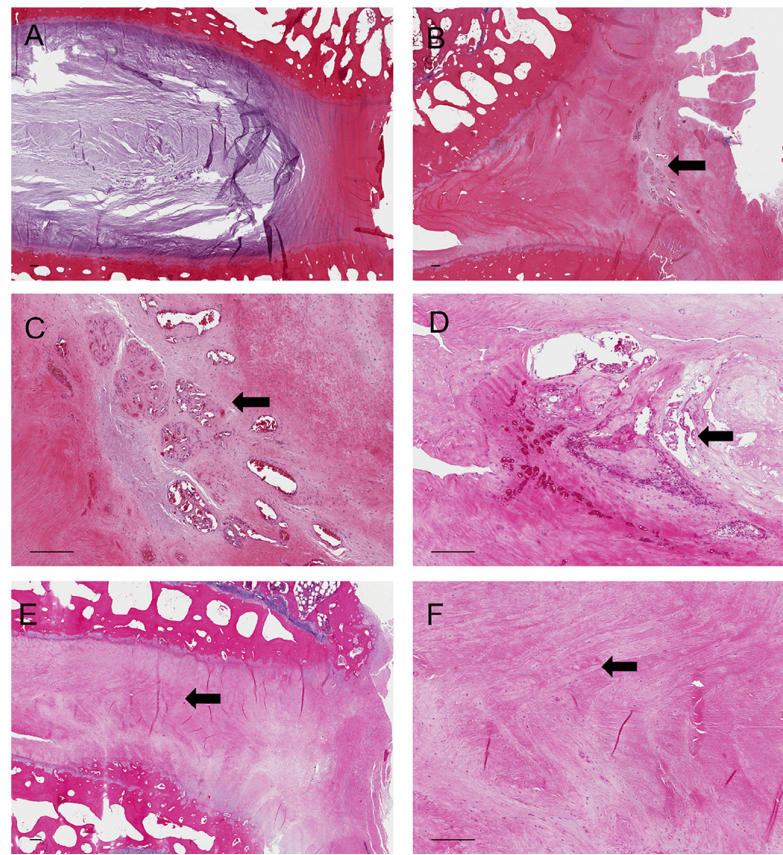
Fig. 7. Sections stained with hematoxylin and eosin. (A) Control disc demonstrating intact AF with adjacent NP. (B) Injured disc with disruption of AF lamellae and vascular proliferation evident (arrow). (C) Injured disc demonstrating vascular proliferation (arrow) under higher magnification. (D) MPC-treated disc with extensive vascular infiltration (arrow). (E) pMPC-treated disc demonstrating lamellar structure (arrow) and reduced vascular infiltration evident relative to B and C. (F) pMPC-treated disc demonstrating lamellar structure (arrow) under high-power magnification. Scale bar=200 um. AF, annulus fibrosus; NP, nucleus pulposus; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell.

consistent with the gross morphology and histologic findings, highlighting the limitations of current radiological imaging techniques for quantitative investigations of the extent of disc injuries and monitoring their repair.

T_{1rho}-weighted MRI, an advanced quantitative MRI method, correlates well with Pfirrmann degenerative grades while also demonstrating a linear correlation between T_{1rho} values and NP glycosaminoglycan content [46]. Discs deemed healthy on Pfirrmann grading demonstrate a wide distribution of T_{1rho} values, suggesting T_{1rho} imaging may represent a more sensitive method for the detection of early degenerative changes [47]. The addition of T_{1rho}-weighted MRI to conventional MRI may aid in the radiological differentiation of early stages of intervertebral disc degeneration in future preclinical and clinical studies to evaluate novel modalities of disc repair.

In the present study, the most significant changes observed in collagen content occurred in the NP1 region of the injured and treated discs where the levels were more than double those of the corresponding normal controls. Apart from the injured and MPC-treated discs, the collagen content of the corresponding AF regions were not significantly different from normal controls. Since each of these injured discs had approximately the same amount of wet weight tissue removed at the time of microdiscectomy, it is clear that the injured disc has the capacity to mount a reparative response. However, it is the nature of the repair tissues which is of importance, particularly with respect to the ability of the substitute to adequately replicate the complex biomechanical function demanded by the activities of spinal movement and weight bearing [48,49]. In this regard, the adult sheep used in our study showed no clinical signs of impaired functions for up to 6 months postsurgery, suggesting that the repair tissue that filled the disc defects was sufficient to maintain their normal functions, notwithstanding their quadrupedal conformation. The morphologic, histologic, and birefringent light microscopy studies provide some insight into the composition and structure of the repair tissue deposited in the injured discs. The non-treated injured group NP1 region contained granulation tissue heavily stained with blood degradation products and exhibited minimal evidence of reconstitution of annular collagen lamellae structure largely
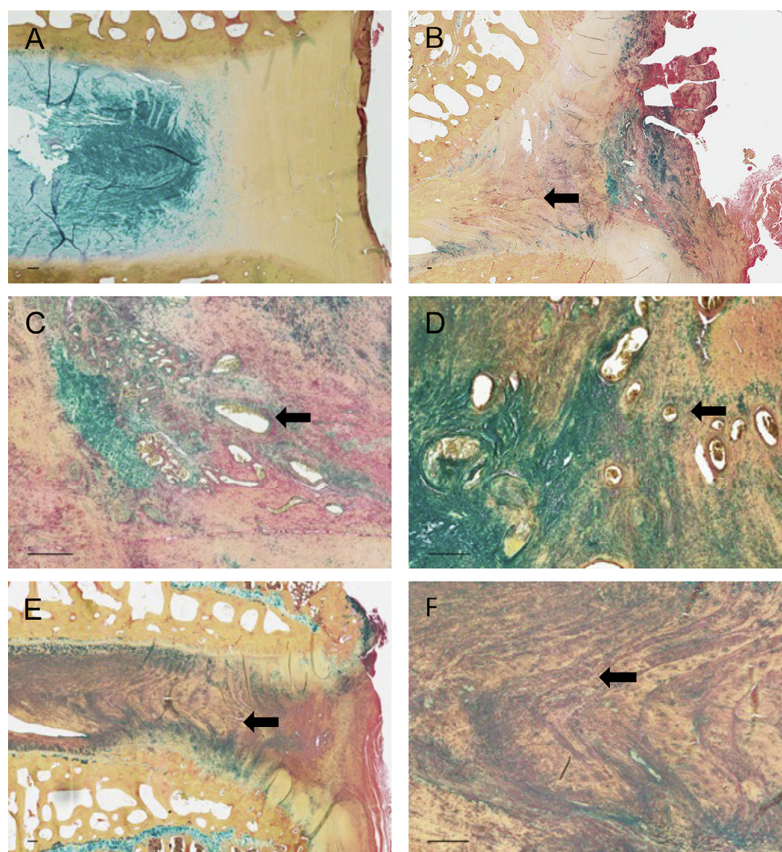
Fig. 8. Sections stained with Alcian blue or Picrosirius red. (A) Control disc demonstrating intact annulus fibrosus with adjacent nucleus pulposus. (B) Injured disc with disruption of AF lamellae evident (arrow). (C) Injured disc demonstrating vascular proliferation (arrow) under higher magnification. (D) MPC-treated disc demonstrating extensive vascular infiltration (arrow). (E) pMPC-treated disc demonstrating lamellar structure of AF (arrow). (F) pMPC-treated disc under higher power demonstrating lamellar structure (arrow) with reduced vascular infiltration relative to injured and MPC-treated discs. Scale bar=200 um. AF, annulus fibrosus; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell.

replaced by primitive granulation or fibrous tissue that provided the high collagen content observed for this region.

The MPC-treated groups exhibited less staining arising from blood degradation products and superior re-organization of the lamellae structure of the AF, particularly for the discs injected with the pMPCs. From these observations we suggest that the presence of MPCs, and pMPCs in particular, within the injured zone of the disc provide the appropriate trophic factors and anti-catabolic mediators to facilitate the endogenous cells to respond to injury by synthesizing an extracellular matrix appropriate for the mechanical demands imposed by normal spinal functions.

The DNA content of a tissue is an imprecise index of its cellularity [50,51]. Nevertheless, the elevation of DNA content of the untreated injured discs reflects the increased cell numbers associated with vascular invasion, elevated monocyte and macrophage infiltration, and the deposition of granulation tissue within the injured discs, as was evident from the histologic and gross morphologic analysis (Figs. 3, 7, and 8) and previous reports [52,53]. Significantly, the DNA content of disc tissues derived from the pMPC-treated group were

observed to be less than the untreated injury group values and equivalent to the normal controls, suggesting partial attenuation of neovascularization and granulation deposition in the presence of the PPS-primed MPCs. It was also observed that although the DNA content of the untreated injured discs was generally higher than the levels in the normal control discs, the injured discs that received the MPCs exhibited a lower content of DNA in the AF2 region. Although this finding could be interpreted to indicate inhibition of endogenous cell proliferation or fragmentation of their DNA because of apoptosis, our previous in vitro studies [34,36] suggest that this was not the explanation. However, additional in vivo studies will be required to resolve this issue.

Our earlier in vitro studies showed that priming of MPCs with PPS upregulated genes encoding aggrecan core protein, insulin-like growth factor 2, and the alpha-chains of type V collagen, whereas the gene encoding the aggrecanase, ADAMTS-4, was downregulated [36]. Although the results of these in vitro studies may not directly translate to the inflammatory environment of the injured intervertebral disc, our earlier microdiscectomy study using the same animal model
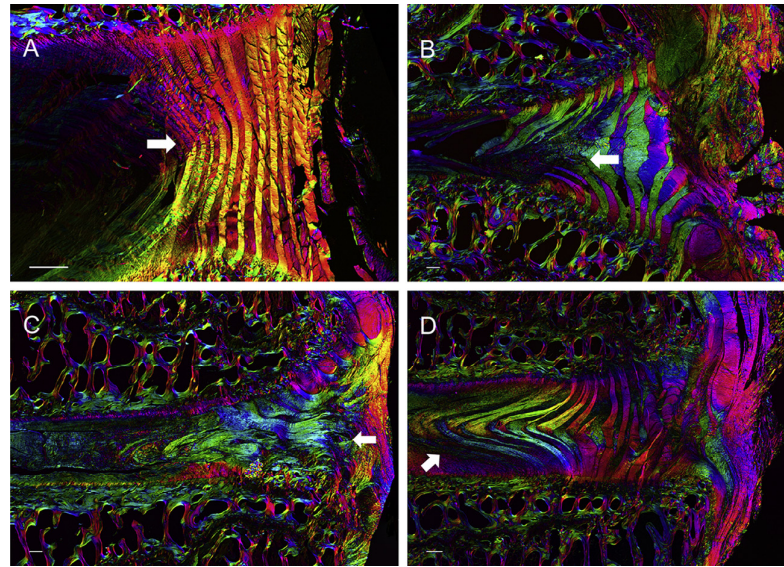
Fig. 9. Birefringent microscopy. (A) Control disc demonstrating intact lamellae of the annulus AF (arrow) adjacent to the NP where the collagen fibers are randomly distributed and do not refract the polarized light. (B) Injured disc showing disruption of AF lamellae structure and the deposition of loose connective tissue (arrow) and vascular proliferation into the AF. (C) MPC-treated disc showing disorganized AF lamellae fragments adjacent to granulomous tissues with blood vessel infiltration (arrow). (D) pMPC-treated disc shows a more organized AF lamellae assembly within the inner AF region and evidence of the re-assembly of the AF fibers in the middle regions. However, outer AF is still a predominately disorganized connective tissue but with reduced vascular infiltration relative to B and C. Original magnification of ×20. Scale bar=500 um. AF, annulus fibrosus; MPC, mesenchymal progenitor cell; pMPC, pentosan polysulfate-primed mesenchymal progenitor cell.

and immunoselected MPC+PPS [8] where PGs from the experimental groups were extracted then subjected to size exclusion chromatography in the presence and absence of hyaluronan (HA) showed that PGs extracted from injured discs implanted with MPC+PPS consisted of a larger population of PG that aggregated with HA than PGs isolated from non-treated injured or normal control discs. This finding indicated that the G1–G2 globular region of the PG subunit core protein was largely intact and thus capable of binding with HA to form the large aggrecan complex [54]. On the basis of this result we speculated that the degradation of aggrecans within the injured discs in our ovine model was mediated by aggrecanases or other proteinases whose activities were attenuated in the presence of the PPS-primed MPCs.

In addition, it is feasible that the strong upregulation of the IGF-2 gene in PPS-primed MPCs [36] once translated to the corresponding protein could, in the hostile environment of injured discs, be supportive of the processes of endogenous disc cell proliferation, differentiation, and extracellular matrix deposition, which again would be supportive of the ongoing tissue repair process [55,56]. However, additional studies will be required to substantiate this suggestion.

Allogeneic bone marrow-derived MPCs were selected for this study because of their high proliferative capacity and retention of phenotype on culture expansion, immunoprivilege, and previous application in other models of disc degeneration [9] and clinical application [31–33]. Moreover, the ability to use allogeneic MSCs as an "off-the-shelf" disc treatment represents a major advantage over autologous therapies,

including chondrocytes from intervertebral disc or immature hyaline cartilage [34]. Although bone marrow-derived MPCs possess osteogenic potential [17], which would represent a distinct problem in the context of intervertebral disc regeneration, published human clinical trials of using allogeneic bone marrow-derived MSCs for the treatment of lumbar intervertebral disc degeneration have not reported such a side effect. Indeed, the cells promoted improvements in pain, disability, and Pfirrmann grade with no evidence of fusion [10]. Mesenchymal stem cells derived from other tissues, such as adipose tissue-derived stromal cells (ADSCs) [10], have been investigated for potential intervertebral disc therapy. ADCSs have demonstrated the ability to promote intervertebral disc regeneration in small and large animal models and produce a PG- and collagen type 1-rich matrix [8]. However, ADSCs required higher levels of transforming growth factors B_2 or additional growth factors to promote chondrogenic differentiation relative to bone marrow-derived MSCs [8]. Interestingly, periodontal ligament MSCs isolated from extracted teeth have been shown to be very responsive to the positive effects of low-dose PPS [57] but have yet to be evaluated in an animal model of disc degeneration.

Limitations

We acknowledge there are several limitations of directly translating the results of the present study to the clinical arena. Firstly, the sheep used in this study, although mature, did not demonstrate degenerative changes on their MRIs before

microdiscectomy. However, the sheep discs, like human discs, have few notochordal cells [58] and has been widely used as a preclinical model for evaluating new therapeutic agents and devices for human spinal applications [8,14,59–61]. A second limitation relates to the anatomical differences between the ovine and the human spines which dictated the surgical approach we were compelled to use in the ovine setting. Traditionally, microdiscectomy in humans is performed via a posterior approach. However, in the sheep spine, because of the extension of the ovine spinal cord into the sacral region and the ossification of the posterior longitudinal ligament, an anterolateral approach was necessary to gain access to the lumbar discs. Furthermore, the experimental time frame of our study was limited to 6 months. Although this postoperative follow-up period may not reflect the long-term outcomes of the pMPC therapy on disc repair following microdiscectomy, we were limited by the ethical and financial constraints of animal experimentation in an academic setting.

Conclusion

Notwithstanding the above limitations, the results of the present study suggest that pMPCs offer a potential modality for limiting the progression of disc degeneration following microdiscectomy.

Acknowledgments

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Chapter 5. Perioperative care for lumbar microdiscectomy: a survey of Australasian Neurosurgeons

5.1. Introduction

Optimization of the discectomy procedure extends beyond the introduction of regenerative therapy at the time of lumbar discectomy. As detailed previously, despite being the most commonly performed spine surgery procedure worldwide, a significant lack of clarity and heterogeneity remains in post-operative care following lumbar microdiscectomy. In order to gain an accurate assessment of the lumbar discectomy peri-operative care practices of Australasian neurosurgeons a survey was conducted with the support of the Neurosurgical Society of Australasia.

This chapter contains a manuscript for a survey study entitled, **"Perioperative care for lumbar microdiskectomy: a survey of Australasian Neurosurgeons."** This manuscript has been accepted for publication by the Journal of Spine Surgery. This chapter describes a survey of Australasian neurosurgeons regarding lumbar microdiscectomy perioperative practices.

The candidate, Chris Daly, contributed to the survey design, conduct and interpretation and the writing of the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xix.

Journal of Spine Surgery

--Manuscript Summary--

Manuscript ID	JSS-17-243
Title	Perioperative care for lumbar microdiskectomy: a survey of Australasian Neurosurgeons
Running Head	Lumbar diskectomy perioperative care
Keywords	Spine,Neurosurgery,diskectomy,perioperative care,surveys and questionnaires
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Section Title	Original Article

Perioperative care for lumbar microdiskectomy: a survey of Australasian Neurosurgeons

Running head: Lumbar diskectomy perioperative care

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Prof. Peter Ghosh contributed to the data interpretation and manuscript revision.

Assoc. Prof. Tony Goldschlager contributed to study conception and design, data interpretation and manuscript revision.

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Abstract

Introduction: Lumbar microdiskectomy is the most commonly performed spine surgery procedure. Over time it has evolved to a minimally invasive procedure. Traditionally patients were advised to restrict activity following lumbar spine surgery. However, post-operative instructions are heterogeneous. The purpose of this report is to assess, by survey, the perioperative care practices of Australasian neurosurgeons in the minimally invasive era.

Methods: A survey was conducted by email invitation sent to all full members of the Neurosurgical Society of Australasia. This consisted of 11 multi-choice questions relating to operative indications, technique, and post-operative instructions for lumbar microdiskectomy answered by an electronically distributed anonymized online survey.

Results: The survey was sent to all Australasian Neurosurgeons. In total, 68 complete responses were received (28.9%). Most surgeons reported they would consider a period of either four to eight weeks (42.7%) or eight to 12 weeks (32.4%) as the minimum duration of radicular pain adequate to offer surgery. Unilateral muscle dissection with unilateral diskectomy was practiced by 76.5%. Operative microscopy was the most commonly employed method of magnification (76.5%). The majority (55.9%) always refer patients to undergo inpatient physiotherapy. Sitting restrictions were advised by 38.3%. Lifting restrictions were advised by 83.8%.

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Discussion: Australasian neurosurgical lumbar microdiskectomy perioperative care practices are generally consistent with international practices and demonstrate a similar degree of heterogeneity. Recommendation of post-operative activity restrictions by Australasian neurosurgeons is still common. This suggests a role for the investigation of the necessity of such restrictions in the era of minimally invasive spine surgery.

Key Words: spine, diskectomy, perioperative care, neurosurgery, surveys and questionnaires

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Introduction

Lumbar microdiskectomy is the most commonly performed spine surgery procedure with over 300,000 operations performed annually in the United States alone(1).

The first lumbar diskectomy for a patient with a pre-operative diagnosis of ruptured intervertebral disk is credited to Mixter and Barr in 1932 and was performed via a multi-level lumbar laminectomy(2). Since the initial description of the lumbar diskectomy procedure the operation has progressed towards its current minimally invasive form. In tandem with the reduction in invasiveness, some surgeons have reduced the restrictions imposed in post-operative care instructions(3). However, significant heterogeneity in surgical technique and perioperative care still exists around the world(4-7) and there is a paucity of literature about the current state of play in Australia.

Understanding current practices would be helpful to patients together with care providers, including referring practitioners, nursing staff, physiotherapists and perhaps other surgeons. The purpose of this survey is to determine current lumbar microdiskectomy perioperative practices amongst Australasian neurosurgeons.

Methods

A survey of Australasian Neurosurgeons was conducted by email invitation sent to all full members of the Neurosurgical Society of Australasia (NSA). All invitees received a further two email reminders. The survey consisted of 11 multi-choice questions conducted by an anonymized online survey. Statistical analysis was performed with Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA) and SPSS Statistics Version 24 (IBM Corp, Amonk, NY, USA). Pearson chi-squared statistic was used to assess the statistical significance of relationships between surgeon seniority and response to variables. Ethics approval was provided by Monash Human Research Ethics Committee.

Results

The survey was sent to 235 Australasian Neurosurgeons (NSA Members). 71 responses were received of which 68 were complete (28.9% complete response rate). Only complete responses were included in the attached report. The questions and results of the survey are detailed below in *Table 1*.

Surgical Procedure

The majority of surgeons (97.1%) performed a unilateral approach; 76.5% of surgeons used a microdiskectomy retractor and 20.6% used a tubular retractor system to perform a unilateral muscle dissection

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with unilateral diskectomy. Only 2.9% of respondents performed bilateral muscle dissection with unilateral diskectomy.

Magnification

The operative microscope was the most commonly employed method of magnification, practiced by 76.5% of surgeons. The operative microscope was used by surgeons performing the unilateral muscle dissection technique with a microdiskectomy retractor (58.8%) and those utilizing a tubular retractor system (17.6%). Loupes were used by 20.6% of surgeons—16.2% using the unilateral approach with microdiskectomy retractor, 2.9% using the tubular retractor system and 1.5% using the bilateral muscle dissection approach. Only 2.9% of surgeons elected to use no magnification and these were evenly split between the bilateral and unilateral muscle dissection approach.

Operative Indications

In the absence of cauda equina syndrome or severe neurological deficit most surgeons reported that they would consider a period of either four to eight weeks (42.7%) or eight to 12 weeks (32.4%) of radicular pain the minimum duration of symptoms before offering surgery. Of the remaining surgeons 4.4% considered less than two weeks appropriate, 11.8% reported two to four weeks and 8.7% deemed more than 12 weeks an acceptable period of radicular pain after which surgery could be offered.

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Post-operative Management

The majority of surgeons (57.4%) mobilized patients upon returning to the ward on the day of surgery. Slightly less than one third of surgeons (32.4%) mobilized patients a few hours after surgery with the remainder (10.2%) mobilizing patients on day one following operation.

Post-operative Physiotherapy

Most surgeons (55.9%) always referred patients to inpatient physiotherapy. Only 13.3% of surgeons state they never referred patients to undergo inpatient physiotherapy. Outpatient physiotherapy referral practices were more varied, with 23.5% of surgeons indicating that they never referred patients for outpatient physiotherapy and 16.2% always referring patients for outpatient physiotherapy. The remaining surgeons fell between these extremes.

*Sitting Restriction*s

Just over one third of surgeons (38.3%) recommended the application of sitting restrictions whilst the remainder (61.7%) recommended no restrictions beyond that afforded by comfort. The breakdown of this advice can be seen in Table 1 and Fig. 1.

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When sitting restrictions were recommended the duration varied between avoiding sitting until comfortable (25%) and four to eight weeks (11.8%). Sitting restrictions of two to four weeks were advised by 17.7% of surgeons. Restrictions of one week or less were rarely recommended (1.5%).

Lifting Restrictions

 Lifting restrictions were advised by the majority of surgeons (83.8%). Lifting restrictions of <5kg (42.6%) and <10kg (39.7%) were most frequently offered. 16.2% of surgeons recommended no lifting restrictions (Fig. 2). One surgeon contacted the authors directly to stress the importance of the avoidance of spinal flexion rather than lifting per se.

A majority of surgeons advised patients to follow lifting restrictions for a period of four to eight weeks (52.9%). The next most common period was two to four weeks (19.1%). The application of restrictions until comfortable was advised by 13.2% of surgeons and no period of lifting restrictions advised by 7.4%. A minority of surgeons advised extension of lifting restrictions beyond eight weeks (5.9%) or for less than one week (7.4%)(Fig. S4)

Seniority and Relationship to Advising Restrictions

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There was a representative range of surgeon experience in this study, with surgeons of less than five years (25.0% of respondents) to more than 20 years of experience (36.8% of respondents) contributing to the survey. Surgeons five years or less post fellowship were more likely to use the operative microscope (94.1%) than those six or more years post fellowship (69.2%). The only surgeons who did not use intraoperative magnification had more than 20 years experience post fellowship. No relationship between surgical seniority and any other variable achieved statistical significance.

Discussion

This is the first survey of Australasian neurosurgeons detailing the peri-operative management of patients undergoing lumbar diskectomy. Australasian practice is generally consistent with international practice and demonstrates a similar degree of heterogeneity (4-7).

A limitation of this study is the 28.9% complete response rate. A trend towards declining responses to clinician surveys has been noted previously(8,9). Reasons cited for this trend include clinicians being "swamped by questionnaires"(10), a lack of incentive for involvement(10) and many clinicians having a policy of non-response (9). Furthermore Australasian clinicians have been noted to be amongst the worst survey responders(8). The response rate is

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comparable to the 36% rate in a survey of members of the
International Society for the Study of the Lumbar Spine regarding
post-operative activity restrictions(11). The distribution of surgeon
seniority in this survey approximates approximated that of the
neurosurgical workforce as described in the 2016 Royal Australasian
College of Surgeons Activities Report suggesting that responders are
representative in this regard(12). The potential for non-responder
bias must, however, be recognized.

Unilateral muscle dissection with unilateral diskectomy is the
approach taken by the majority of Australasian neurosurgeons and is
consistent with the reported practices of Dutch spine surgeons(4).
The nature of the operative approach preferred by British, Canadian
and Italian surgeons was not reported.

Concurrent with the trend towards reduced soft tissue dissection in
lumbar diskectomy increased use of operative magnification has also
been practiced (13). The operating microscope was introduced to
lumbar diskectomy surgery by Yasargil in 1967 and reported as part of
a series of patients in 1977(14). Its use was subsequently
popularized by the pioneering orthopaedic spine surgeon John
McCulloch(13,15) and is the practice of over three quarters of
Australasian neurosurgeons surveyed. Canadian neurosurgeons
report a similar practice with 70% using the microscope(5). Similarly,

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82.5% of neurosurgeons from Lombardy reported using either loupes or the operative microscope (the two were not distinguished in the Italian survey) while 17.5% reported no use of magnification, much higher than the 3% in our survey. The figures for Dutch and British surgeons were not reported.

Variation among spine surgeons regarding timing of surgery in patients with radicular pain has been noted in previous studies(4). The majority of Australasian neurosurgeons surveyed consider a period of four to eight weeks (42.65%) or eight to twelve weeks (32.35%) the minimum period of radicular pain a patient must report prior to being offered lumbar diskectomy surgery in the absence of cauda equina syndrome. These figures are very similar to those reported by Dutch spine surgeons– 34% of surgeons indicated they would offer surgery to patients with four to eight weeks of symptoms and a further 42% would offer surgery at eight to 12 weeks (4).

Timing of surgery for lumbar disk herniation associated sciatica is a long-standing controversy amongst spine surgeons. Surgical practice has swung from periods of early surgical intervention for acute radicular pain (16) to the current approach favoured by most surgeons of performing a trial of conservative management lasting a number of weeks. The evidence from randomized controlled trials suggests early surgical intervention may offer symptomatic benefit.

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On as-treated analysis, the Spine Patient Outcomes Research Trial (SPORT)(17,18), demonstrated statistically significant benefits to surgery at all time-points from three months to four years(17,18). Similarly, the Sciatica Trial, comparing early (six to 14 weeks) surgery for sciatica with six months of conservative management and surgery thereafter if required, demonstrated faster pain relief and perceived recovery in those who received early surgery(19).

Just over half of Australian neurosurgeons always prescribe post-operative inpatient physiotherapy with less, only one in six, always prescribing outpatient physiotherapy. International practice regarding physiotherapy referral varied significantly: 85.7% of Dutch surgeons prescribed inpatient physiotherapy while only 6% of Italian neurosurgeons did the same. This reflects the lack of clarity regarding the role of post-operative physiotherapy following lumbar microdiskectomy. A recent Cochrane review of post-operative rehabilitation programs following lumbar microdiskectomy indicated that there is, at best, low level evidence that physiotherapy programs started four to six weeks post-surgery led to better function than no treatment(20).

Post-operative activity restrictions are commonly imposed following lumbar diskectomy. This can be observed in our survey and a survey of international surgeons specifically addressing lifting restrictions

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following spine surgery(11).   Our survey results reflect the

persistence of this practice with approximately 40% of Australasian

neurosurgeons recommending specific sitting restrictions. A survey of

British spine surgeons indicated that 31% request their patients do

not sit for between two days to six weeks following a spine surgical

procedure(6). The British survey does not provide further detail on

sitting restrictions and data for post-operative sitting restrictions were

not available for Dutch, Italian or Canadian Spine Surgeons.

Lifting restrictions were more common, with specific lifting restrictions

advised by the majority of Australasian neurosurgeons (83.2%).

Approximately half of Australasian neurosurgeons advised the

application of such restrictions for a period of four to eight weeks.

The recommendation for lifting restrictions is relatively consistent

with the practice among British spine surgeons and surgeons of the

International Society for the Study of Lumbar Spine Surgery, 85%(6)

and 96.3%(11) of whom respectively advised lifting restrictions(6).

The variety of lifting restrictions demonstrated in this survey are

consistent with earlier reports of European spine surgeon

recommendations in which the authors noted a lack of consistency.

(11)

The underlying rationale for sitting and lifting restrictions is that

following surgery the spine is weaker due to disruption of the

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functional spinal motion unit and thus potentially more prone to
injury(11).   However, there appears to be little evidence to support
this theory, empirically or biomechanically, or the imposition of such
restrictions(11,21). The purpose of such restrictions in the era of
microdiskectomy has been questioned in the literature since at least
the mid 1990s(3,22). Carragee et. al. reported a prospective study of
152 patients who did not observe activity restrictions following lumbar
microdiskectomy.   This cohort achieved similar outcomes, with the
exception of achieving earlier return to work, compared to literature
reported outcomes for the standard practice of post-operative
restrictions. Bono et. al.(23) recently reported the first randomized
controlled trial comparing post-operative activity restriction protocols
following lumbar microdiskectomy. No significant differences in
outcomes or disk herniation recurrence rates were observed with
activity restriction protocols of two or six weeks. The study was,
however, underpowered to detect a significant difference in
reherniation rates. We are currently performing the first randomized
controlled trial comparing a patient group observing no post-
operative activity restrictions with a control group observing a one
month period of activity restrictions(24). Additionally, our study will
track patient adherence to activity restrictions following spine surgery
through use of a wearable activity monitory, an innovation of
importance given the difficulty of verifying patient adherence to
activity restrictions through self-report.

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Conclusion

In conclusion, the surgical techniques and magnification preference of Australian neurosurgeons are generally consistent with their international colleagues. Sitting and lifting restrictions are still frequently advised by Australasian neurosurgeons. This survey demonstrates the heterogeneity in peri-operative practices of Australasian neurosurgeons and suggests the potential for investigation of the role of post-operative activity restrictions following lumbar microdiskectomy given the prevalence and variety of these practices in the era of minimally invasive spine surgery.

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Disclosure Statements

The authors have no potential conflicts of interest to disclose

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Tables

Table 1. Survey questions and results. The table lists all survey questions, responses and the percentage of surgeons who selected each response.

Figures

Figure 1   **Fig. 1**   **Sitting restrictions**. Percentage of

surgeons.
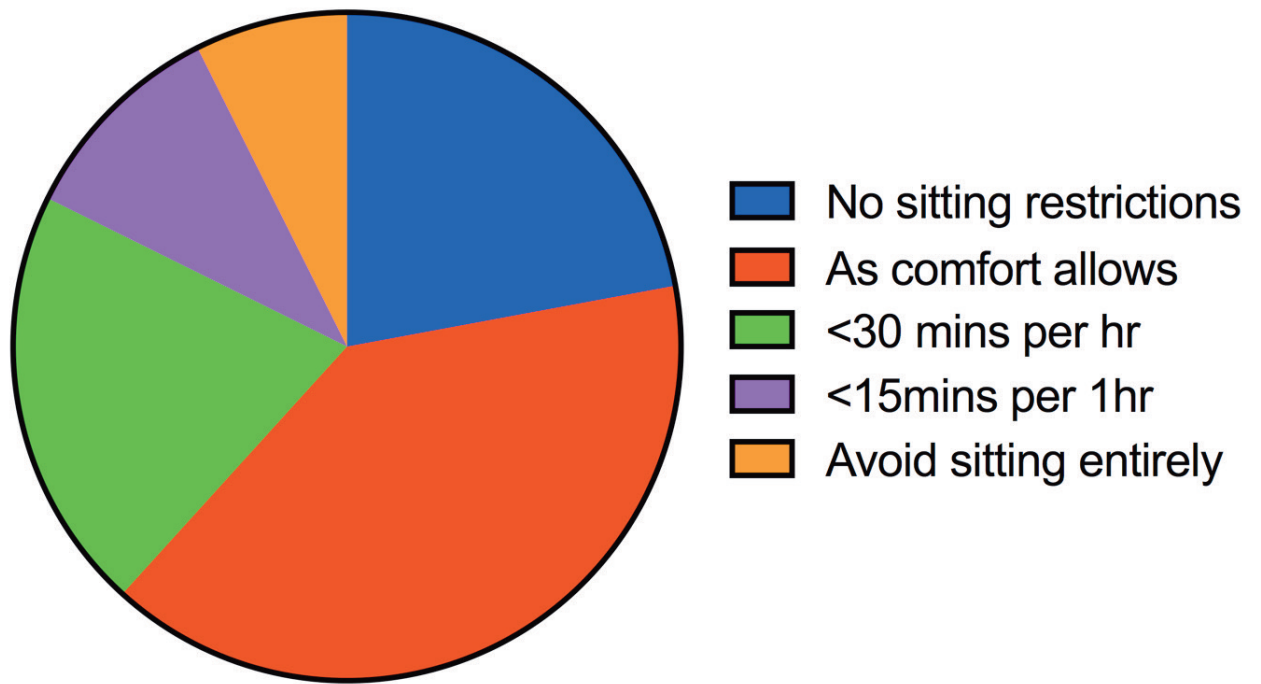
Figure 2

Lifting Restrictions. Percentage of surgeons.
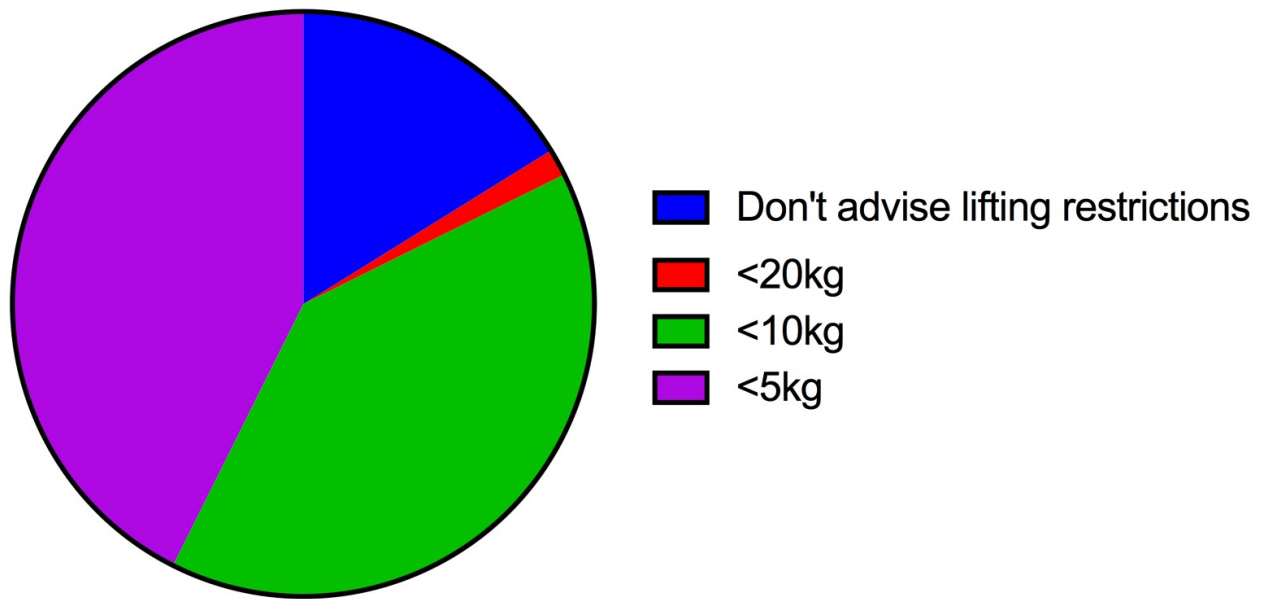
Table 1. Survey questions and results. The table lists all survey questions, responses and the percentage of surgeons who selected each response.

Question	Response	Percentage
1. **Which surgical technique do you perform as a standard procedure for lumbar diskectomy?**	Bilateral muscle dissection with bilateral diskectomy	0.0%
	Bilateral muscle dissection with unilateral diskectomy	2.9%
	Unilateral muscle dissection with unilateral diskectomy	76.5%
	Unilateral muscle dissection with unilateral diskectomy via tubular system	20.6%
	Bilateral muscle dissection with bilateral diskectomy	0.0%
2. **Do you use magnification when performing lumbar diskectomy operations and if so what kind?**	No magnification	2.9%
	Loupes	20.6%
	Microscope	76.5%
3. **In the absence of cauda equina syndrome or severe neurological deficit, what is the minimum duration of radicular pain a patient must report for you to offer lumbar diskectomy surgery?**	<2 weeks	4.4%
	2-4 weeks	11.8%
	4-8 weeks	42.7%
	8-12 weeks	32.4%
	>12 weeks	8.7%
4. **In the absence of CSF leak when do you allow your lumbar diskectomy patients to mobilize following their operation?**	Day 0, upon returning to the ward	57.4%
	Day 0, after a few hours	32.4%
	Day 1	10.2%
	Day 2	0.0%

5. Do you prescribe inpatient post-operative physiotherapy during admission following lumbar diskectomy?	Never	13.2%
	Rarely	8.8%
	Sometime	8.8%
	Often	13.3%
	Always	55.9%
6. Do you prescribe postoperative outpatient physiotherapy after diskharge following lumbar diskectomy?	Never	23.5%
	Rarely	17.7%
	Sometime	35.2%
	Often	7.4%
	Always	16.2%
7. Do you advise sitting restrictions in the post-operative period following lumbar diskectomy?	No sitting restrictions	22.0%
	As comfort allows	39.7%
	<30 mins per hour	20.6%
	<15 mins per hour	10.3%
	Avoid sitting entirely if possible	7.4%
8. How long after lumbar diskectomy do you advise patients to restrict sitting time?	I don't advise sitting restrictions	30.9%
	Until comfortable	25.0%
	<1 week	1.4%
	1-2 weeks	13.2%
	2-4 weeks	17.7%
	4-8 weeks	11.8%
	>8 weeks	0.0%
9. Do you advise lifting restrictions in the post-operative period?	I don't advise lifting restrictions	16.2%
	<40kg	0.0%

	<20kg	1.5%
	<10kg	39.6%
	<5kg	42.7%
10. How long after microdiskectomy do you advise patients to restrict lifting?	No lifting restrictions	7.4%
	Until comfortable	13.2%
	<1 week	1.5%
	2-4 weeks	19.1%
	4-8 weeks	52.9%
	>8 weeks	5.9%
11. How many years have you been practicing as a Consultant Neurosurgeon?	0-5 years	25.0%
	6-10 years	19.1%
	11-15 years	5.9%
	16-20 years	13.2%
	>20 years	36.8%

Chapter 6. Lumbar microdiscectomy and post-operative activity restrictions: a protocol for a single blinded randomised controlled trial

6.1. Introduction

This chapter contains a manuscript for an experimental study entitled, **"Lumbar microdiscectomy and post-operative activity restrictions: a protocol for a single blinded randomised controlled trial."**. This manuscript has been published in BMC Musculoskeletal Disorders. This chapter describes the protocol of a single blinded randomised controlled trial comparing post-operative regimens consisting of one month of activity restrictions with an equivalent period with no activity restrictions. In addition, an interim analysis of recruitment to date is provided.

The candidate, Chris Daly, contributed to the trial design, conduct and the writing of the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xix.

6.2. Impact of post-operative activity restriction on lumbar microdiscectomy clinical outcome: a single blinded randomised controlled trial *interim analysis*

Recruitment of patients in the post-operative activity restriction on lumbar microdiscectomy clinical outcome trial commenced in February 2016.

A total of 106 patients were enrolled with 89 undergoing randomisation. Six patients did not undergo surgery, eight patients withdrew and three were ineligible. The first patient underwent surgery on 5/2/2016. Median follow-up of enrolled patients is 7.8 months at the time of completion of this interim analysis. This data is presented in **Figure 1**.

To date five patients have MRI confirmed intervertebral disc reherniation. Three underwent revision lumbar microdiscectomy, one received a nerve root block and the final patient was treated with conservative management.

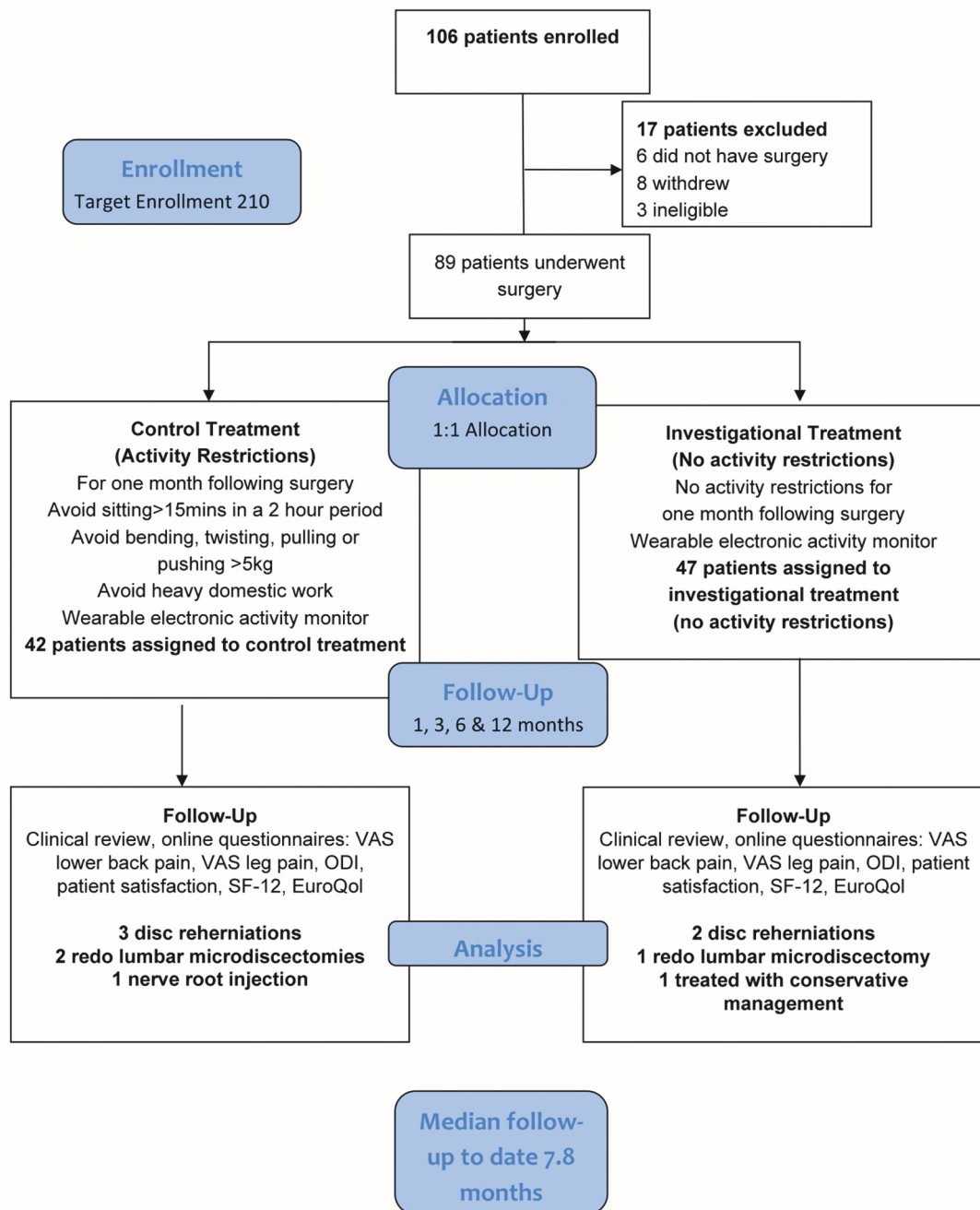The target enrolment for the trial is 210 patients and is anticipated to be completed in 2020.

Figure 1. Lumbar microdiscectomy and post-operative activity restrictions trial flow diagram

BMC Musculoskeletal Disorders

Lumbar microdiscectomy and post-operative activity restrictions: a protocol for a single blinded randomised controlled trial

Chris D. Daly[1,2,3*], Kai Zheong Lim[1,2], Jennifer Lewis[2], Kelly Saber[4], Mohammed Molla[2], Naor Bar-Zeev[5] and Tony Goldschlager[1,2,3,6]

Abstract

Background: Lumbar microdiscectomy is the most commonly performed spinal surgery procedure, with over 300,000 cases performed annually in the United States alone. Traditionally, patients were advised to restrict post-operative activity as this was believed to reduce the risk of disc reherniation and progressive instability. However, this practice would often delay patients return to work. In contemporary practice many surgeons do not restrict patient post-operative activity due to the perception this practice is unnecessary. We describe a randomised controlled trial to assess the impact of activity restrictions on clinical outcome following lumbar discectomy.

Methods/Design: The lumbar microdiscectomy and post-operative activity restriction trial is a multi-centre, randomised, controlled single blinded trial. Two hundred ten patients due to undergo single level lumbar microdiscectomy without a history of previous spine surgery, infection or fracture are randomised to be advised either restricted or unrestricted activity for a period of 30 days following lumbar microdiscectomy. Actual adherence with trial allocation will be monitored bioelectronically via a wearable device. Outcome assessment at follow up will occur at 1, 3, 6 and 12 months. The primary outcome will be a composite endpoint comprising changes in Visual Analogue Scale (Leg and Back), Oswestry Disability Index and the absence of intervertebral disc reherniation or secondary intervention.

Discussion: This randomised controlled trial will directly compare post-operative protocols of activity restrictions and no restrictions following lumbar discectomy with adherence monitored bioelectronically.

Trial Registration: Australian New Zealand Clinical Trials Registry: ACTRN12616001360404 (retrospectively registered 30/09/2016).

Keywords: Lumbar Discectomy, Spine, Restrictions, Post-Operative, Sitting

Background

Lumbar microdiscectomy is the most commonly performed spinal surgical procedure [1]. Lumbar microdiscectomy is indicated for radicular pain unresponsive to conservative management (e.g. analgesia and physiotherapy), neurological deficit (e.g. weakness) or for cauda equina syndrome.

Lumbar microdiscectomy is minimally invasive, patients typically mobilize the same day and are discharged home the following day, making the operation suitable for day-procedure [2]. Traditionally following surgery, patients have been advised to restrict sitting, lifting or resuming other activities of everyday life, and are advised to either stand or lie for variable periods [3]. Sitting imposes greater intradiscal pressure than does standing [4] though evidence that increased pressure increases disc reherniation risk is lacking.

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Such restrictions impact upon patients' ability to return to work, travel or drive and basic comfort. It has been suggested that activity restrictions may also raise patient anxiety regarding reherniation risk. Moreover, neurosurgical practice regarding activity restriction varies, the dearth of evidence resulting in absence of clear clinical guidelines for surgeons, nurses, physiotherapists and occupational physicians. If no difference in outcomes are observed between groups in this randomised controlled trial future patients would be able to rapidly resume their normal activities, productivity, work and do so without fear or associated psychological morbidity. This would provide an evidence base to postoperative care and consensus amongst surgeons.

Two prospective studies published in the 1990s reported incidence of symptomatic recurrent disc protrusions and reoperation, and time to return to work in a cohort of patients whose movement was not restricted post lumbar microdiscectomy. Compared to rates in the literature among movement-restricted patients, adverse outcomes in this cohort were not considered higher [5, 6]. However, in the absence of a control group and randomisation, the evidence from such studies is relatively weak.

Bono et al. [7] recently published the first report of a randomised controlled trial investigating post-operative activity restrictions following lumbar discectomy. This trial compared post-operative protocols consisting of short (two weeks) and long (six weeks) periods of activity restriction following lumbar discectomy. The authors observed no significant difference in outcome as assessed by Visual Analog Scale (VAS) back or leg pain or Oswestry Disability Index (ODI). Disc reherniation rates differed between the groups observing short (11%) and long (7%) periods of activity restriction. This difference did not achieve statistical significance or translate into an appreciable difference in clinical outcome. However, the authors noted the study was underpowered to detect a significant difference in disc reherniation rate and calculated approximately 800 patients per arm would be required to achieve sufficient statistical power.

All previous studies on post-operative restrictions following lumbar discectomy have relied on self-reported adherence to mobility restrictions. Non-adherence is a well-recognized phenomena in spine surgery trials with non-adherence rates in SPORT approximately 40% at one year [8]. Such outcomes are likely to be biased. Contemporary wearable electronic devices that can accurately record the patient's position (i.e. sitting/standing) enable empirical observation of patient adherence to a regimen of sitting restrictions with great reliability. This trial will be the first to track post-operative adherence to activity restrictions following lumbar discectomy and the impact of adherence on outcomes.

Methods

Question
Is the outcome of patients without restrictions inferior to those observing sitting and activity restrictions following lumbar discectomy?

Objectives
The study aims to determine whether the outcome of patients without imposed sitting and other behavioral restrictions post lumbar microdiscectomy are inferior to those of patients with imposed restrictions, in terms of disc reherniation, pain and disability outcome measures.

Design
The will be a randomised controlled surgeon and assessor-blinded trial. The trial design is illustrated in Fig. 1.

Hypothesis to be tested
That movement restriction following first episode lumbar microdiscectomy in adults results in improved outcomes in disc reherniation rates, pain and disability outcome measures.

Participants
The study will consist of patients aged 18–75 years old who meet the inclusion criteria and are undergoing first-episode lumbar microdiscectomy for symptomatic lumbar disc prolapse in the participating private and public hospitals in Melbourne, Victoria, Australia during the trial period. Informed consent will be sought.

Inclusion Criteria.

Participants will be

1. Age 18–75 years
2. Suffering from radiculopathy or radicular pain with concordant MRI evidence of lumbar disc herniation at L3/4, L4/5 or L5/S1

Exclusion criteria

1. Previous history of lumbar surgery, spinal infection or spinal fracture.

Trial sites
The trial is a multi-centre trial conducted in three hospital in Melbourne, Victoria, Australia. The hospitals are as follows:

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Fig. 1 Lumbar microdiscectomy and post-operative activity restrictions trial flow diagram
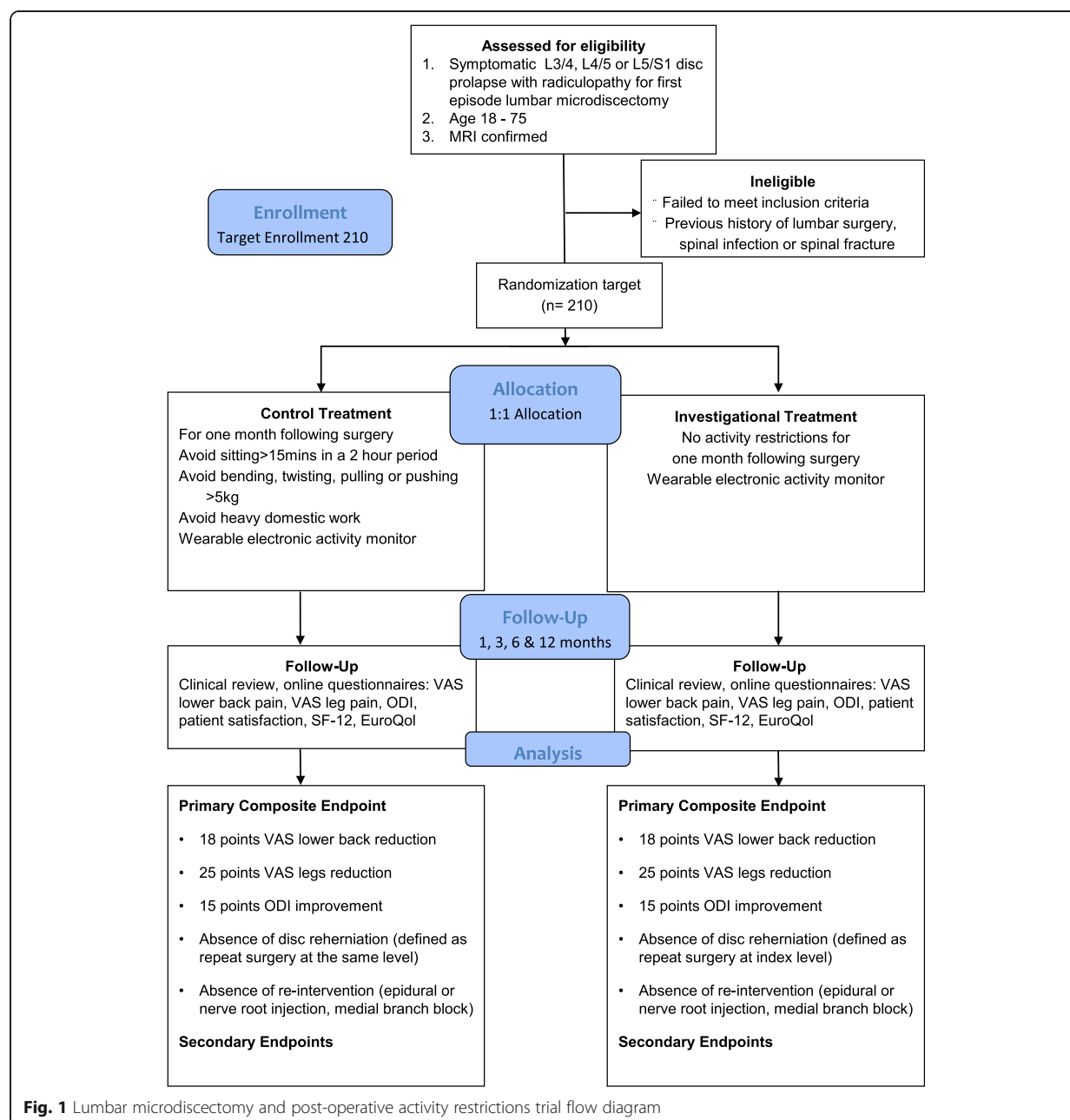
1. Monash Medical Centre, Clayton, Melbourne, Victoria, Australia
2. Jessie McPherson Private Hospital, Clayton, Melbourne, Victoria
3. Cabrini Hospital Malvern, Malvern, Melbourne, Victoria, Australia

Treatment allocation Patients will be randomised to one of two parallel treatment arms allocated in a 1:1 ratio. Sealed numbered envelopes containing electronically randomised group allocations and group specific post-

operative activity instructions will be prepared prior to trial commencement. Following informed consent, a sealed pre-randomised envelope will be allocated by the study nurse to the patient and the patient label affixed to the envelope. The envelope will then be handed to the treating physiotherapist to be opened postoperatively. The study interventions are specifically detailed on this instruction sheet and will be read by the physiotherapist to the patient postoperatively. For both allocation groups, the study card instructions are discussed and reinforced by the treating physiotherapist. The

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physiotherapist also gives the patient the activity monitor and instructs them on how to use this. The patient will receive a copy of the instruction card to take home and is advised not to disclose their allocation group to medical staff or assessors. The physiotherapist is not involved in subsequent assessment of the patient.

Post-operatively both groups will be fitted with electronic monitoring devices, worn on either the thigh under clothing or carried in the pocket, that will record patient position (sitting/lying/standing) and activity (walking/running/cycling). The devices will be taken off when showering or bathing.

Control Treatment

Post-operative activity restrictions represent the traditional standard of care following lumbar microdiscectomy. As such the control group will be advised to follow post-operative activity restrictions for a period of one month following lumbar microdiscectomy. The control group will receive the following specific advice:

For the first one month following surgery:

1. Avoid sitting for longer than 15–30 min in any two hour period
2. No bending, lifting, twisting, pulling or pushing greater than 5 kg
3. Avoid heavy domestic work such as vacuuming, laundry and making beds

And for the first two weeks following surgery:

4. Avoid strenuous sexual activity

The restrictions detailed above reflect post-operative algorithms in current clinical practice. [unpublished data, Daly et al.].

Investigational treatment

The investigational treatment arm will be the group without sitting or other restrictions. They will be advised to return to normal activities with no restrictions placed on sitting, exercise, return to work, or other activities as soon as they feel ready.

Outcomes

Primary endpoints

While there is no widely used definition of clinical success following lumbar microdiscectomy it is generally accepted that such a definition should take account of outcome measures such as physical function, disability and pain [9–11]. Intervertebral disc reherniation and reoperation are important considerations as potential primary endpoints. In SPORT[12] 20% of patients who underwent surgery rated their progress

as less than a major improvement at one year yet only 6% had undergone reoperation. Thus, reherniation and reoperation alone do not account for the majority of unsatisfactory patient outcomes following lumbar discectomy. The use of a composite endpoint allows for the capture of multiple outcomes that influence the overall success of a clinical intervention while also allowing for increased statistical efficiency and efficient resource utilisation [13, 14].

As such the primary endpoint to be assessed in this trial consists of a composite of the following widely accepted outcome measures:

1. 18 point reduction in VAS lower back
2. 25 point reduction in VAS legs
3. 15 point improvement in ODI score
4. Absence of disc reherniation (defined as repeat surgery at the same level)
5. No other secondary intervention (epidural or nerve root injection, medial branch block)

Using the above definitions of treatment success we would anticipate treatment success rates of ~70–90% in keeping with those reported in the literature [8, 15]. This allows the ability to detect a clinically significant difference in outcomes between the two groups (i.e. rates of clinical success) with smaller groups than required for the detection of differences in recurrence rates (i.e. event rate of "clinical success" of approximately 70–90% as opposed to herniation event rate of 5–10%).

Utilisation of the personal wearable electronic device will enable accurate assessment of patient adherence to the allocated post-operative care group.

Secondary endpoints

Secondary endpoints will consist of the following surgical and functional endpoints.

Surgical endpoints:

1. Incidence to 12 months post-operatively of disc reherniation requiring repeat surgery at the same level
2. Incidence to 12 months post-operatively of other parenteral pain management intervention such as epidural or nerve root injection, medial branch block for the primary illness, but excluding enteral or dermal analgesia (fentanyl patches, TENS machine or acupuncture).

Functional endpoints:

1. VAS lower back change score
2. VAS legs change score
3. ODI change score

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4. Days to return to work (including days to return to modified duties and to normal duties.)

For functional endpoints, magnitude of change from baseline, adjusted for baseline score will be compared between treatment groups. Additionally, the proportion achieving predefined success thresholds (change scores of 18 for VAS back, 25 for VAS legs and 15 for ODI) will be compared.

Duration of treatment

Patients will be instructed to follow the post-operative advice- i.e. restrictions or no restrictions for a period of one month. Monitoring of adherence using wearable electronic activity monitors (Activ8, 2 M Engineering, Netherlands) will be for 1 month.

Follow up schedule

With the assistance of a blinded investigator, participants will complete online outcome questionnaires preoperatively, the day following surgery, and at home at one, three, six and 12 postoperative months.

Questionnaires will include:

1. VAS lower back pain
2. VAS leg pain
3. ODI
4. Current situation/patient satisfaction questionnaire
5. SF-12/EuroQol (EQ-5D)(quality of life questionnaire)

Patients will receive standard post-operative trial-blinded neurosurgical outpatient review at approximately 30–60 days following surgery at trial sites. Teleconsultation will occur at the three, six and 12 months. Patients will only receive further neurosurgical outpatient review if clinically indicated.

Participant timeline

The participant timeline is illustrated below in Table 1.

Randomisation and allocation concealment

Randomisation of treatment protocol to sequentially numbered envelopes was performed by an electronic randomisation tool. Sealed envelopes will be sequentially assigned immediately following consent. The treating physiotherapist is given a sealed envelope by the blinded study nurse upon randomisation. This contains the patients assigned group and the appropriate post-operative instructions. This is opened by the physiotherapist post operatively.

Blinding

Blinding will be universal from consent till surgery. Post-operatively the physiotherapist will open the envelope and inform the patient of their assigned group. The study nurse will complete all assessment of patients at each time point and will be blinded as to patient randomisation throughout the study. Participants will be instructed not to inform study staff regarding their allocation. Surgical staff will remain blinded throughout the trial.

Non-Adherence

Patient data will be analysed on an intention to treat basis. Non-adherence to post-operative activity protocol may be determined by analysis of recorded activity from the wearable device. This will be especially important in light of the trial explaining to participants as part of consent procedures the clinical uncertainty regarding movement restriction, which may result in poor adherence in the group subsequently assigned to restriction.

Sample size

As detailed earlier one randomised controlled trial investigating the role of post-operative activity restrictions in outcome following microdiscectomy has recently been reported [7]. The authors recruited 108 patients and noted disc reherniation rates of 11% in the 2-week restriction group and 9% in the 6-week restriction group. Previous studies have indicated a reherniation rate ranging from 2 to 18% [16]. Annual reported reherniation rates have been closer to 4–5% in large series [8, 17, 18]. The authors of the randomised controlled trial calculated it would be necessary to have 800 patients in each arm in order to detect a statistically significant difference in disc reherniation rate and that this may not be feasible. We are in agreement with this assessment.

Reported rates of clinical success for lumbar discectomy vary widely dependent on the criteria. Using the criteria detailed in our composite primary outcome we would anticipate a clinical success rate within the broad range reported in the literature of approximately 70–90%.

In determining the power of this study, we assume that approximately 80% of patients will meet the definition of treatment success. The calculation of sample size can be based upon a threshold of a 20% difference in treatment success as clinically significant (i.e. 80% success vs. 64% success). In order to have 80% power to detect a 20% difference in the binomial outcome of treatment success defined at $p = 0.05$ the sample size calculated would be 78 patients per group. If we allow for an approximately 30% drop-out rate, this will bring the calculated sample size to 105 patients per group for a total of 210 patients in the trial.

Table 1 Participant timeline template for schedule of enrolment, interventions and assessment

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
TIMEPOINT	*Post-clinic review*	*Pre-operative*	*Pre-operative Survey*	*Day 1*	*1 Month*	*3 Months*	*6 Months*	*12 Months*
ENROLMENT:								
Eligibility screen	X							
Informed consent	X							
Allocation		X						
INTERVENTIONS:								
[Post-Operative Restrictions Group]				←———→				
[Control Group]				←———→				
ASSESSMENTS:								
[Pre-operative Survey)]			X					
[Clinical Review]					X	X	X	X
[Post-operative Surveys]				X	X	X	X	X

Analyses

Baseline characteristics

The baseline characteristics of patients and operative details will be recorded.

Statistical analysis

Hazard of reherniation and of parenteral analgesia will be compared by treatment arms. Efficacy will be defined as 1 minus hazard ratio of active vs restricted arms. Efficacy less than absolute delta (see sample size) will be deemed equivalent. Change from baseline adjusted for baseline in functional scores will be compared across treatment arms. Magnitude difference will be compared using ranksum (Mann-Whitney-U) test.

Analysis will report both intention to treat and per protocol results. Per protocol adherence to treatment will compare time in movement by treatment arm using t-test allowing for differential variance. Time in movement should differ between arms. Two thresholds will be defined a priori – a sedentary level below which will be considered adherent to movement restriction, and an activity level above which non-restriction will be deemed to have occurred. These thresholds will be used to define adherence. In sensitivity analysis we will examine impact on trial outcomes of excluding those subjects allocated to restriction who moved above this threshold and those unrestricted who were sedentary. We will also conduct sensitivity analysis by cross allocating such subjects.

Discussion

The longstanding practice of applying post-operative activity restrictions following lumbar spine surgery was based upon the hypothesis that such restrictions may reduce the risk of progressive instability or lumbar disc reherniation [5]. Furthermore, prolonged sitting has been suggested to decrease lumbar lordosis, increase spinal loading and muscle activity and contribute to accelerated disc degeneration and low back pain independent of previous operative intervention [19]. The randomised controlled trial of Bono et al. [7] demonstrated no significant difference in outcome measures between patients who observed two weeks or six weeks of activity restrictions though was underpowered to detect differences in reherniation rates. The only prior studies investigating the impact of removing post-operative restrictions reported no increased risk of reherniation or reoperation in patients not observing activity restrictions following lumbar discectomy surgery but these studies lacked a comparator group and their design was subject to potential bias [5, 6]. Modern lumbar discectomy is now minimally invasive and results in less tissue destruction, further undermining the hypothetical rationale for activity restriction. In a recent survey of Australasian Neurosurgeons, many advised either no sitting restrictions (22%) or sitting as comfort allows (40%) [unpublished data, Daly et al.]. However, the vast majority (84%) advised restricted lifting.

In an uncontrolled prospective cohort study [6] patients who did not observe post-operative activity restrictions returned to work earlier. Mean time to return to work in the cohort was 1.2 weeks. Currently extant recommendations suggest four to 16 weeks off work following lumbar discectomy surgery.

Activity monitoring/adherence

The activity monitor will monitor and record patient posture (i.e. lying, sitting or standing) and activity (walking, running or cycling) over the one month

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period following lumbar microdiscectomy. This device is sensitive to acceleration. The validation report described 90.8% correlation between activity monitor output and video analysis [20]. Data is recorded over the one month period on the stand-alone device and then transferred to the study computer.

Conclusion

Back pain is the leading cause of disability worldwide [21] and intervertebral disc degeneration is a significant contributor to back pain. Lumbar microdiscectomy, performed for symptomatic intervertebral disc herniation, is the most commonly performed spine surgical procedure. Activity restrictions have traditionally been recommended following this operation and patients often advised to delay return to work for four or more weeks with restrictions of similar duration applied to other activities of daily life [6]. Clarification of the role of post-operative restrictions will allow standardisation of post-operative care and potentially allow patients to return to work more rapidly thus reducing the social and economic burden of this condition.

Availability of data and materials
Not applicable. This manuscript does not contain any data.

Authors' contributions
CDD: study design, conduct of study, analysis of data and corresponding author. KZL: development of online study instruments and analysis of data. JL: study design, conduct of study, data collection, patient management. KS: study design, patient management. MM: study design. NBZ: study design and analysis, manuscript revision. TG: study conception, design, initiation, conduct, analysis and grant holder.

Ethics approval and consent to participate
Ethics approval has been granted by Monash Health Human Research Ethics Committee Ref: 15379L. All subjects consented to participate in this trial.

Consent for publication
Not applicable.

Competing interests
NBZ has received investigator initiated project grants from GSK Biologicals and from Takeda Pharmaceuticals.

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Chapter 7.   General Discussion

Lumbar discectomy remains the most commonly performed spine surgery procedure worldwide(1) and is successful in relieving symptoms and signs of neural compression in greater than 80% of patients(2). However, it fails to halt the underlying process of disc degeneration. As a result up to one third of patients progress to suffer back pain(3), which is disabling in up to 10%(4), 12% will undergo reoperation within four years and up to 40% of these patient will undergo fusion surgery(5). Thus, despite the efficacy of lumbar discectomy surgery in relieving radicular pain, the importance of improving the long-term outcome of this procedure, in an attempt to minimise disc reherniation, chronic low back pain and further surgery, is abundantly clear. Furthermore, despite being the most commonly performed spine surgical procedure, post operative care practice following lumbar discectomy remains markedly heterogenous(6-9). There is a particular lack of clarity surrounding post operative activity restrictions, which was first raised over 20 years ago(10). It is unclear if post operative restrictions minimise recurrent herniation, or alternatively, unnecessarily contribute to delayed patient return to work. The objective of this thesis is to attempt to address the shortcomings of lumbar microdiscectomy surgery in contemporary practice and thus aim to "re-engineer" this operation. A significant contributor to the unfavourable clinical sequelae of lumbar discectomy surgery is the failure of conventional lumbar discectomy surgery to address underlying disc degeneration. There is a real need to develop a therapy, which preferably can be administered at the time of microdiscectomy, to promote intervertebral disc regeneration of the injured disc. The first step in developing such a therapy for the post discectomy intervertebral disc is to have an appropriate animal model. As such the preclinical component of this thesis consisted of the development of an appropriate animal model of the post lumbar discectomy intervertebral disc and investigation and development of a novel cellular therapy for intervertebral disc degeneration to be delivered at the time of lumbar discectomy. These studies are described in experimental Chapters 3 and 4.

The thesis then changes focus to improving contemporary lumbar microdiscectomy surgery by providing evidence into the best post operative clinical management of this patient cohort. Specifically, clarification of the role, if any, of post operative activity restrictions following lumbar discectomy is studied. In an attempt to understand current practice, a survey of Australasian neurosurgeons was performed in Chapter 5. This led to the development of a multicentre randomised controlled single blinded clinical trial comparing a post operative protocols of one month of activity restriction with a protocol of no activity restrictions. This is detailed in Chapter 6.

Numerous animal models of lumbar intervertebral disc degeneration exist(11), however, relatively few large animal models of the post discectomy intervertebral disc have been developed. The sheep has

many desirable qualities as an animal model of intervertebral disc degeneration, including the lack of notochordal cells in the adult intervertebral disc(12), similarities in size to the human intervertebral disc and exposure to biomechanical similarities in spite of its quadrupedal stature(13).

Numerous methods exist for inducing intervertebral disc degeneration in animal models (11,14-18). The features of the post discectomy intervertebral disc are distinct, resulting from a combination of intervertebral disc herniation and the surgical procedure. This particular scenario is not represented in most existing large animal models of disc degeneration. The disruption that occurs to the post microdiscectomy intervertebral disc is characterized by both an annular and nuclear defect. The defect in the nucleus and annulus has important mechanical consequences that impact on both the ability of the disc to tolerate and respond to mechanical challenges(19) and to the prospect of applying regenerative therapies to the intervertebral disc. Particular challenges that may present in the context of applying regenerative therapies include the higher potential of leakage from the annular defect(20) and the reduced nuclear contents, notably nucleus pulposus cells and proteoglycans. Given that one potential mechanism of action of cellular therapy is by effecting the local milieu and influencing the behaviour of resident cells(21), the nature of the population of remaining resident cells bears particular consideration.

The two injury models, studied in this thesis, both induce a full thickness annular injury extending into the nucleus that would produce a consistent injury and defect. The established ovine annulotomy injury model of Osti(16) consists of a partial thickness annular incision and is commonly used in studies of intervertebral disc degeneration. However, it fails to replicate the unique anatomical challenges of the post discectomy intervertebral disc. The discectomy annulotomy model as described by Oehme et al.(22), in which a full thickness annular incision is created followed by removal of 200mg annulus fibrosis and nucleus pulposus, was selected partly due to its obvious similarity to the lumbar discectomy procedure performed clinically. The drill bit injury model of Zhang et al.(23) also demonstrated similarities to the lumbar discectomy procedure by inducing a full thickness annular injury extending into the nucleus. The drill injury method offered the potential additional benefit of providing an injury that was technically easier to replicate.

Comparison of these two models revealed the discectomy annulotomy model produced a greater degree of degeneration after six months than the drill bit injury model as assessed by disc height index changes, gross morphological, biochemical and histological analysis. Biochemical analysis demonstrated a significant reduction in nucleus pulposus and overall glycosaminoglycan content, with an associated increase in collagen content, in the discectomy annulotomy injured discs relative to the drill injured discs. Intra-operatively and at the time of necropsy the annular defect produced by the drill bit injury method was often not readily apparent whilst the discectomy annulotomy defect was clearly

evident at both time points. This can be appreciated in the gross morphological images in Fig. 4, page 41, of the included manuscript in Chapter 3. The histological finding of neurovascular invasion in the discectomy annulotomy injured discs, which was absent in the drill injured disc, is also supportive of the appropriateness of the discectomy annulotomy model given similar findings in herniated and degenerative intervertebral discs clinically(24,25). This neurovascular invasion of degenerate lumbar intervertebral discs is hypothesized to be one of the mechanisms responsible for back pain(24). Given the incidence and morbidity of chronic low back pain following lumbar discectomy the importance of neurovascular invasion in the discectomy annulotomy model cannot be overstated.

3T and 9.4T MRI Pfirrmann grades did not demonstrate any significant difference in outcome between these two injury models although both models produced significantly greater degeneration than normal controls. The discrepancy observed between the radiological analysis and other measures were attributed to the relative lack of sensitivity of the qualitative radiological analysis, relative to the higher degree of quantitative precision afforded by biochemical and disc height index analysis. This discrepancy between the results of qualitative radiological analysis and quantitative biochemical analysis has been observed in previous studies(22,26). Our group is currently researching methods to take full advantage of the superior resolution afforded by 9.4T MRI to better differentiate among stages of early disc degeneration(27).

The results of this study indicated that the discectomy annulotomy injury model produced an injury more consistent with that of lumbar discectomy surgery with a clear annular and nuclear defect. This defect, in turn, also afforded opportunities for the administration of therapy at the time of the injury. In comparison, the drill bit injury model produced a less severe injury, extent of degeneration and also did not provide a defect affording administration of regenerative therapy at the surgical site, as would be the case following clinical lumbar discectomy. The drill bit injury method may provide an appropriate model of spontaneous disc degeneration with nuclear herniation, however, this remains to be explored.

Although the two injury models discussed were considered to replicate closest the post lumbar discectomy intervertebral disc, neither are without shortcomings. As a quadrupedal animal model the ovine discectomy annulotomy model cannot completely replicate the bipedal intervertebral disc. Non-human primate models of intervertebral disc degeneration have been described(28,29), and although such animals are quadrupedal for locomotion they do spend significant amounts of time in an erect posture, and as such may better model the bipedal human. An annulotomy injury performed on the intervertebral discs of such animals may afford a better large animal model of the post-discectomy intervertebral disc, however ethical and financial constraints would likely preclude such investigation in many institutions. The inability of either ovine model to allow an assessment of low back pain, the adverse clinical outcome most commonly experienced by patients following lumbar discectomy, is also

a significant limiting factor. Animal models of low back pain have been described, however, only for small animals to date(30-32). Translating findings from rodent models of intervertebral disc degeneration to the clinic is fraught with limitations due to presence of notochordal cells, and significant differences in anatomy and mechanical properties. These considerations noted, the ovine lumbar discectomy annulotomy model still represented one of the best available models of the post ovine lumbar intervertebral disc and as such was selected for the investigation in the regenerative studies described in Chapter 4.

The failure of conventional therapy to adequately address intervertebral disc degeneration has led to extensive efforts by investigators globally to develop biological therapies for this condition(33). Cell therapy has been identified as possessing particular promise in this application and this has been demonstrated in multiple preclinical(22,26) and clinical studies(34-37) throughout the world. A multitude of cells have been investigated for this application including autologous disc chondrocytes (35,38), haematopoietic stem cells(39), embryonic stem cells(40) and induced pluripotent stem cells(41). However, the majority of investigators and reports to date have focused on two broad categories of cells, chondrocytes and mesenchymal stem cells.

Chondrocytes are the intuitive choice as these are the cells present within the intervertebral disc that produce and maintain the proteoglycan rich extracellular matrix responsible for many of the mechanical properties of the disc. The potential of this approach has been repeatedly demonstrated in preclinical and clinical studies. Radiological and clinical outcome benefits have been demonstrated in patients with correlative biochemical, histological and radiological benefits observed in animal models(42,43). Furthermore, this therapy has been clinically available for many years in Germany although yet to receive FDA approval(44).

However, the autologous disc chondrocyte approach has numerous disadvantages that reduce the likelihood that this approach will gain widespread acceptance. The requirement for harvest of cells from a diseased disc prior to administration to the degenerate disc limits the procedure to patients undergoing surgical intervention for one disc with another degenerate painful disc. The harvested cells must then be expanded in Good Manufacturing Practice (GMP) facilities. As such facilities are expensive, the application of this procedure may be limited to only large centres. Furthermore, the harvested cells are derived from a diseased disc and as such are likely to have reduced efficacy and a degree of senescence(45). Following cell culture and expansion these cells must be administered at a separate procedure. It is these limitations that increase the attractiveness of MSCs for application in intervertebral disc regeneration.

MSCs offer major advantages by way of their immunoprivilege(46) and thus have the ability to be used allogeneically, in an "off the shelf" capacity. This ability enables cells from young healthy donors to be used that may have greater functionality than those of the often older patient if such cells were autologous in origin(47). A further benefit of this "off-the-shelf" utilization is enabling the administration of cellular therapy as a single stage procedure, in contrast to the separate, metachronous, cell harvest and administration procedures necessary for autologous approaches. Mesenchymal progenitor cells are an immunoselected pure cell population that possess this same immunoprivilege while also demonstrating increased clonogenic, development and proliferative capacity compared with unfractionated MSCs(48).

Our group has previously demonstrated that MPCs were able to promote intervertebral disc regeneration three months post administration of chondroitinase-ABC(17). However, the cABC model does not replicate the post-discectomy intervertebral disc, as degeneration occurs due to enzymatic degradation of chondroitin sulfate chains of the PGs, but not the collagenous or cellular components of the disc, that are affected by an annular and nuclear defect. In a subsequent study MPCs combined with pentosan polysulfate (PPS) were administered twelve weeks following partial thickness annular incision (26). Discs injected with MPC combined with PPS recovered disc height and their mean DHI was significantly greater than the DHI of controls with PPS alone and nil-injected discs. This demonstrated the ability of MPC combined with PPS to promote intervertebral disc regeneration in an animal model of disc degeneration induced by annular injury. However, again, this did not specifically replicate the post discectomy intervertebral disc.

The pilot study of Oehme et. al (22) demonstrated the ability of a combination of MPC and PPS in a gelatin scaffold to promote intervertebral disc regeneration in an ovine discectomy annulotomy model, in which a full thickness annulotomy was performed with removal of 200mg of annular and nucleus pulposus tissue. The MPC and PPS treated discs demonstrated significant improvements in disc height index, MRI Pfirrmann grades, proteoglycan content and histological analysis at six months compared to untreated discs. Whilst providing some positive results, this pilot study was performed on only six sheep and lacked a MPC only group, precluding analysis as to the effect of MPC exposure to PPS on disc regeneration.

In previous in vitro studies PPS has been demonstrated to promote MPC proliferation and chondrogenic differentiation(49). Additionally, in vitro priming of MPCs with PPS in culture for 24-48 hrs was demonstrated to promote upregulation of pathways for chondrogenic differentiation, proliferation and proteoglycan production(50). Priming of MPCs with PPS potentially affords regulatory advantages as it represents a manufacturing method rather than requiring co-administration of MPCs and PPS. Given the promising results of both the pilot study and the in vitro priming experiment a definitive study

investigating the potential of PPS primed MPCs to promote intervertebral disc regeneration following lumbar discectomy was required. The inclusion of a treatment group receiving unprimed MPCs would enable analysis of the effect of PPS priming of MPCs on the intervertebral disc regeneration.

This definitive study is described in Chapter 4. Following annulotomy discectomy injury, sheep were randomised into three groups: the injury group (which received no further therapy), the MPC group (which received 0.5×10^6 unprimed MPCs in a gelatin matrix with fibrin sealant) and the pMPC group (which received 0.5×10^6 PPS primed MPCs in a gelatin matrix with fibrin sealant). The study described in chapter 4 demonstrated that both PPS primed MPCs (pMPC) and MPCs, when inserted into the disc space in a gelatin matrix with fibrin sealant, promoted intervertebral disc regeneration compared to injured discs. However, the pMPC treated discs demonstrated evidence of superior regeneration as assessed by gross morphological, biochemical, histological and birefringent microscopic analysis. The pMPC treated discs demonstrated reduced gross morphological injury scores relative to the injured untreated discs with much less nuclear discolouration. The pMPC discs also demonstrated significantly increased AF and NP GAG content with reduced DNA content relative to the MPC treated discs. These findings correlated with reduced vascular infiltration and increased structural organization observed in the pMPC treated discs relative to MPC treated discs under histologic and birefringent microscopic analysis. The correlation of these quantitative and qualitative findings strongly suggested that the PPS priming of MPCS afforded additional benefits to the promotion of intervertebral disc regeneration.

The observation that DNA content and vascular invasion of disc tissues derived from the pMPC-treated group was less than in the injury group suggests attenuation of neovascularization and scar tissue formation by the PPS primed MPCs. In this regard, it is noteworthy that our previous in vitro studies on pMPCs revealed the down-regulation of the surface antigen, CD146, that has recently been shown to be a high-affinity receptor for Netrin-1(51). This protein is a neuronal guidance molecule that promotes angiogenesis and vascular development of endothelium by interaction with CD146 receptors on endothelial cells(51). Given the decreased neovascularization in the pMPC-treated group, we speculate that CD146 down-regulation in pMPCs may be a contributing factor to this process, however additional studies will be required to confirm this suggestion.

The invasion of nerves accompanying blood vessels is one of the hypothesized mechanisms for pain generation in discogenic back pain(24,52). The absence of vascular invasion in the pMPC treated discs may also correlate with reduced neural invasion and back pain. The animal models used in this model, unfortunately, as discussed previously, did not model back pain. Only small animal models to date have been able to provide some information on pain(30,31). Due to inherent difficulties translating findings of pain from small animal models to the human intervertebral disc, the observation of reduced

vascular invasion and neural invasion needs to be tested in a clinical trial where pain and disability can be properly assessed.

Priming of MSCs to promote specific phenotypes that support a particular clinical application is not new(53). Interferon-gamma(54) or interleukin-17(55) are used to enhance immune suppression, interleukin-1 to promote an anti-inflammatory phenotype(56) and dexamethasone and transforming growth factor-ß three to promote chondrogenesis(57). Priming of MSCs towards a nucleus pulposus chondrocyte like phenotype has previously been reported with growth differentiation factor 6 and transforming growth factor-ß(58), FGF-2 and TGF-ß(59), hypoxic culture conditions(60) and culture in nucleus pulposus cell-derived acellular matrix(61). Hypoxic conditioning of mesenchymal stem cells during two-dimensional culture expansion and three-dimensional culture has been demonstrated to promote improved mechanical properties and increased glycosaminoglycan and collagen content in the nucleus pulposus of tissue engineered intervertebral discs(62). In a pilot clinical study five patients who received hypoxic culture expanded autologous mesenchymal stem cells for low back pain reported overall improvement, strength improvement and four reported improvements in mobility(63). This study shows the potential for the clinical application of priming of MSCs and MPCs in the context of promoting intervertebral disc regeneration, although larger randomised studies are required. Priming of MPCs with PPS is one of the few priming approaches with demonstrated efficacy in the promotion of intervertebral disc regeneration in a large animal model. PPS also has a long standing history of safe clinical utilization(64), in contrast to growth factors such as TGF-ß with known oncogenic potential(65). Furthermore, PPS provides a significant economic advantage as a cheap and clinically readily available drug compared to the relatively expensive growth factors cited in many other studies.

The mechanism by which MPCs implanted into the intervertebral disc space effect disc regeneration remains the subject of some conjecture. There are three major hypothesized mechanisms of action. The first is local survival of the implanted MPCs with differentiation towards chondrogenic cells. Such cells would produce proteoglycan and other extracellular matrix components and thereby aid in the restoration of the structure of the intervertebral disc, in which the resident cells have limited capacity. Studies to date have had varying results with regard to demonstrating persistence and differentiation of implanted mesenchymal stem cells into the intervertebral disc. Henrikson et al.(66) demonstrated xenogeneic MSCs administered to porcine discs differentiated into cells resembling chondrocytes as determined by expression of aggrecan, collagen II, sox-9 and these cells had the capacity for disc matrix production. These xenogeneic cells persisted in the intervertebral disc at time points of up to six months. Autologous MSCs have also been demonstrated to differentiate towards a nucleus pulposus phenotype on implantation into the disc(67-69). However, Acosta et al.(70), and other investigators, have questioned the ability of implanted cells to remain viable within the avascular intervertebral disc

and demonstrated a lack of viable MSCs from three months onwards, following implantation in a porcine model(70). In a recent study utilizing MSCs co-labelled with a Positon Emission Tomography (PET) reporter gene and magnetic iron oxide nanoparticles, MSC viability in the intervertebral disc could not be demonstrated from four weeks onwards, despite the confirmation of the presence of cells in the disc(71). It is possible that the number of implanted cells could ultimately influence their survival in the intervertebral disc's hostile environment(72). Of interest, in the xenogeneic porcine study of Henriksson et al.(66), MSCs survived for up to six months, but porcine intervertebral discs were injected with a lower dose of 0.5×10^6 MSCs. In the allogeneic MSC study of Acosta et al.(70), in which cell survival could not be demonstrated beyond three weeks, porcine intervertebral discs received 25×10^6 MSCs. In a study specifically assessing cell number as a determinant of cell viability Serigano et al.(73) demonstrated that administration of 10^6 MSCs lead to superior cell viability and extracellular matrix production relative to 10^5 or 10^7 cells. Hence, the influence of cell count must be taken into account in studies of stem cell survival following transplantation. The cell count used in the studies in chapter 4 was approximately 0.5×10^6, similar to the recommendations of Serigano et al.(73) in the porcine model. There is mixed evidence supporting the chondrogenic differentiation of MSC following transplantation, however, it is likely that other significant mechanisms of action are involved, as will be discussed below.

The second major hypothesized mechanism of action is localized release of factors from the MSCs influencing the local milieu and resident cells(21). MSCs and MPCs secrete cytokines and growth factors that may enhance NP proliferation, cell matrix synthesis and suppress mechanically mediated disc degeneration (74,75). MSCs can also prevent notochordal cells or NP progenitors from undergoing apoptosis. Yang et al.(69) reported inhibition of disc cell apoptosis and disc matrix repair by transplanting autologous MSCs, further supporting the paracrine mechanism of action theory. The paracrine hypothesis has even lead some investigators to propose the administration of MSC released vesicles, rather than MSCs, as a potential method of providing the therapeutic effects of MSCs without the administration of cells(21). A recent study demonstrated that bone marrow derived MSC exosomes, defined as "extracellular vesicles that are released from cells upon fusion of an intermediate endocytic compartment, the multivesicular body, with the plasma membrane"(76) typically containing numerous contents, such as cytokines, proteins, lipids, mRNA, which promote nucleus pulposus cell proliferation and expression of matrix proteins(75). In the same study, the reverse was also true as nucleus pulposus cell exosomes induced bone marrow derived MSC migration and differentiation towards an NP-like cell lineage(75). Such findings are supportive that MSCs promote intervertebral disc regeneration via paracrine mechanisms in addition to differentiation.

The third major hypothesized mechanism of action is that MSCs have anti-inflammatory properties(77). A number of studies have reported the ability of MSCs to modulate the inflammation and immune

system functions in vitro and in vivo(78). MSCs have been shown to produce anti-inflammatory cytokines and inhibit pro-inflammatory cytokine production, it has been demonstrated that they can produce a reduction of inflammation associated cartilage destruction in animal models of arthritis (79-82). A recent study demonstrated the ability of MSC conditioned medium to downregulate the expression of proinflammatory cytokines such as interlekukin-1ß (IL-1ß), IL-6 and tumour necrosis factor (TNF) produced in the pathogenesis of discogenic pain in an in vitro model of the degenerate disc(83). Furthermore, MSCs have been demonstrated to have additional effects that may provide benefit in disc degeneration such as down regulation of primary nociceptive afferent input with consequent improvement of chronic pain(84,85). Ultimately, further studies utilizing assays for the determination of the presence, differentiation and viability of cells in the intervertebral disc, and cytokine profiling need to be conducted in the future in order to determine the relative contribution of differentiation of MPCs, interaction with the local milieu and modulation of inflammation as mechanisms of intervertebral disc regeneration.

The post microdiscectomy intervertebral disc presents additional challenges for cellular therapy. The presence of annular and nuclear defects increases the potential for cell leakage. Omlor et al. (86) reported that only 9% of transplanted MSC cells remain in the disc three days after injection in a porcine nucleotomized intervertebral disc model. Vadala et al.(20) found no evidence of GFP labelled MSCs in the disc in a rabbit model of disc degeneration at nine weeks, and concluded that cell leakage contributed to the development of peripheral osteophytes. No evidence of heterotopic ossification was observed in our studies. Furthermore, the evidence of significant regeneration in both the MPC and pMPC treated discs relative to the injured discs suggest at least the persistence of sufficient biologically active material to facilitate regeneration, as no evidence of regeneration was observed in the injured untreated discs. Additional studies utilizing iron oxide nanoparticle labelled MPCs are being conducted by our group to further investigate the fate of these cells.

Ultimately, the efficacy of MPC transplantation into the intervertebral disc must be demonstrated in clinical trials for this promising therapy to progress. Phase II clinical trials of percutaneous intradiscal MPC injection to treat discogenic back pain have been completed and demonstrated significant reduction in pain, disability, opiate consumption and radiological translational movement of the disc in MPC treated patients relative to controls(34). Phase III trials are currently under way investigating this therapy. We are currently designing a modification of these clinical trials to specifically suit the microdiscectomy scenario. The aim is to treat the disc with PPS primed MPCs in a gelatin matrix with fibrin sealant at the time of the microdiscectomy operation. The other components are already in clinical use and as the MPCs have been used in other clinical trials without safety concerns, we are hopeful of ethical approval. Specifically, PPS is currently used to treat interstitial cystitis(87) and the

gelatin matrix and fibrin glue are used routinely in surgery. This clinical study is an exciting direct translation of this thesis and will provide important safety and efficacy data evaluating the potential of this therapy to target this unmet clinical need in this large patient group.

In addition to optimizing lumbar microdiscectomy surgery by developing a cellular therapy to promote intervertebral disc regeneration this thesis also sought to optimize the post operative care of lumbar discectomy and thus facilitate patient recovery. The first lumbar discectomy for a patient with a pre-operative diagnosis of ruptured intervertebral disc is credited to Mixter and Bar in 1932 and was performed via a multi-level laminectomy(88). Since that time the operation has evolved to its current minimally invasive form in which patients undergo the operation with the aid of microscopic visualization and will be discharged home the same day in many centres. Activity restrictions are advised for varying periods following discectomy surgery due to the hypothesis that such restrictions may reduce the risk of lumbar spine instability or reherniation of the treated intervertebral discs(10). In tandem with the evolution of the surgical procedure towards its current minimally invasive form, some surgeons have reduced the requirement for post operative restrictions. As a result there is significant heterogeneity in post operative practices with no Level I evidence to support either the application or absence of post operative activity restrictions at the time of commencement of this thesis(10,89).

In order to better understand the current heterogeneity in practice this thesis sought to first gain an appreciation of perioperative practices amongst Australasian neurosurgeons through the performance of an online survey via the Neurosurgical Society of Australasia(90). The results of this survey are detailed in chapter 5. The major findings of this survey, with relevance to post operative care and activity restrictions, were that Australasian neurosurgeons demonstrated a similar degree of heterogeneity in post operative practices compared to their international counterparts. Sitting restrictions were advised by 38.3% of Australasian neurosurgeon and lifting restrictions advised by 83.8%. These results were similar to those of British spine surgeons for sitting restrictions and lifting restrictions(6). Heterogeneity in post operative lifting recommendations were also observed among members of the International Society for Study of the Lumbar Spine(91). This emphasized the need for a well designed prospective clinical trial to answer this question.

Prior to commencement of this thesis there was an absence of randomised controlled trials investigating the role of post operative activity restrictions following discectomy surgery. In the 1990s Carragee et al.(10,89) reported the outcome of a prospective clinical trial in which 152 patients did not follow activity restrictions following lumbar discectomy. This cohort demonstrated outcomes comparable to those reported in the literature for patients following traditional post operative advice with regard to sciatica, disc reherniation (11%) and reoperation (5%) over a minimum of two years of follow-up. Furthermore, one third of this cohort returned to work within one week of their surgery. This study

suggested the safety of the removal of activity restrictions following lumbar discectomy and the potential role for a randomised controlled trial in the future.

A randomised controlled trial was designed, approved and implemented in this thesis to investigate the role, if any, of such restrictions. The protocol of this trial and the interim analysis is provided in Chapter 6. Following the commencement of the trial described in Chapter 6, Bono et al.(92) released the results of their randomised controlled trial comparing two weeks of activity restrictions with six weeks of activity restrictions following lumbar microdiscectomy. This study suggested equivalent clinical outcomes irrespective of the length of post operative activity restrictions as assessed by VAS back pain, VAS leg pain and ODI scores up to one year of follow-up. Although, by the authors own admission, underpowered to detect a significant difference in reherniation rate, with a total of 108 patients recruited, the authors did note a difference in this rate with 6 (11%) of patients in the two week restrictions group and 4 (7%) of patients in the six week restriction group experiencing intervertebral disc reherniation. Further analysis indicated that recruitment of over 800 patients in each group would be required in order to detect a statistically significant difference in reherniation rate which, as the authors commented, may not be feasible. An additional technical challenge associated with this trial is the reliance on patient self-report to determine adherence to the activity regimen.

The protocol described in chapter 6 attempts to overcome some of the limitations encountered by Bono et. al. and benefited from the input of Professor Bono as a reviewer of the protocol as part of the publication process. The inclusion of an activity monitor that reports on patient posture and activity, i.e. sitting, standing, lying, walking, running or cycling, will enable conclusions to be drawn regarding the role of activity restrictions with greater confidence in the adherence of patients to the prescribed regimen. Patient non-adherence is a well-recognized phenomenon within the spine surgery literature. Non-adherence rates exceeding 40% for interventions as significant as surgical vs. nonsurgical management have been observed(2). All studies to date on post operative activity restrictions have been reliant on patient self report to determine adherence to post operative instructions. The inclusion of an activity monitor will dramatically increase the power of this randomised clinical trial to arrive at meaningful conclusions regarding the role of post operative activity restrictions. Furthermore, the use of a composite endpoint (93-95), will allow a meaningful analysis of outcome to be performed with a smaller sample size than the 800 patients per allocation group required for a statistically significant analysis of reherniation rates. Composite endpoints provide a mechanism to incorporate multiple outcomes in a trial in which there is no obvious choice of one primary outcome while also allowing for increased statistical efficiency and efficient resource utilisation (95,96). To date, the trial has recruited just over 100 patients with a target enrolment of 210. Given the 12 month follow-up and recruitment rate to date the trial is anticipated to be completed in 2020. Upon completion and analysis this trial will

provide Level I evidence regarding the role of postoperative activity restrictions following lumbar microdiscectomy thus affording the opportunity to standardize care following the most commonly performed spine surgical procedure. Significantly, the inclusion of the activity monitor will allow meaningful analysis of the relationship between actual post operative patient activity and outcome, rather than mere self-reported activity.

7.1. Conclusion

Stem cells are one of the most promising therapies in development for the treatment of intervertebral disc degeneration. Phase II clinical studies have already demonstrated the potential of percutaneously delivered mesenchymal progenitor cells to promote intervertebral disc regeneration in the context of discogenic back pain (34) and Phase III studies are underway. Furthermore, clinical pilot studies have demonstrated the ability of autologous and allogeneic mesenchymal stem cells to promote improvements in patient pain and disability with associated radiological improvements(97,98). The study of the effects of PPS priming on MPCs reported in this thesis indicate that PPS priming promotes superior intervertebral disc regeneration to MPCs alone. The beneficial effects of PPS priming of MPCs were demonstrated on gross morphological, biochemical and histological analysis. Successful translation of this therapy presents an opportunity to dramatically improve the outcome of patients who would otherwise suffer progressive disc degeneration and its attendant sequelae following lumbar microdiscectomy.

The most common adverse clinical sequelae following lumbar discectomy is chronic low back pain(3). Unfortunately, low back pain is not adequately assessed in available large animal models. Small animal model studies have demonstrated that MSCs have efficacy in producing long-term pain relief in persistent pain models with possible mechanisms including interaction with endogenous opioid receptors and down-regulation of primary afferent nociceptive input(84,85). Such findings strongly suggest clinical MPC therapy may provide additional therapeutic benefit beyond those measurable in large animal studies. A clinical trial to investigate PPS primed MPCs delivered at the time of lumbar microdiscectomy will commence in the near future and will offer the ability to investigate the efficacy of this therapy and its potential to improve the outcome of patients following lumbar microdiscectomy, including the incidence and morbidity of low back pain.

Conventional MRI failed to appreciate the significant differences between the MPC and pMPC treated discs evident on biochemical and histological analysis in the study in chapter 4. This reflects the insensitivity of conventional MRI in assessing early disc degeneration relative to the quantitative biochemical analysis and the subtle gradations in degeneration that could be appreciated on gross morphological and histological analysis. Our group had demonstrated the potential of the application of

high-resolution 9.4T MRI scanners in differentiating stages of early intervertebral disc degeneration(27). Such advanced imaging technologies are powerful tools in the investigation of the pathogenesis of disc degeneration and following clinical translation and adoption ultimately may provide greater diagnostic accuracy through enabling visualization of neurovascular invasion of the intervertebral disc (99). This is the subject of ongoing investigation by our group.

Re-engineering of lumbar microdiscectomy surgery extends beyond the development of novel biological therapies. The heterogeneity of post operative activity advice presents an incredible opportunity to positively impact the recovery and quality of life of patients following this very common procedure. The survey of Australasian neurosurgeons reported in chapter 5 demonstrated that marked heterogeneity in post operative care practices was present within even this relatively small neurosurgical community and was consistent with the global experience(7,8). The completion of an appropriately designed, adequately powered, single blinded, multicentre randomised controlled trial to address the role of post operative activity restrictions following lumbar microdiscectomy will produce Level I evidence regarding the impact of such restrictions on patient outcome and return to work. The novel innovation of inclusion of an activity tracker will ensure that patient outcomes in this trial may be correlated with the activity protocols observed and not merely the protocol advised.

In summary, this thesis presented a novel cellular therapy for the treatment of disc degeneration at the time of lumbar microdiscectomy comprised of pentosan polysulfate primed mesenchymal progenitor cells in a gelatin matrix with fibrin sealant. This novel therapy demonstrated exceptional potential in an ovine model of the post lumbar discectomy intervertebral disc and is readably translatable, hence a pilot clinical study is planned in the near future. This thesis presented a single blinded multicentre randomised controlled trial to provide Level 1 evidence on the role of post operative activity restrictions following lumbar microdiscectomy. The completion of this trial has the potential to allow standardization of post operative activity advice following lumbar microdiscectomy surgery on the foundation of Level I evidence for the first time. The ultimate translation and widespread clinical application of this novel cellular therapy and the standardization of post operative activity advice has the potential to re-engineer lumbar microdiscectomy surgery.

7.2. References

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Appendix 1. Pentosan Polysulfate Binds to STRO-1^{+} Mesenchymal Progenitor Cells, is Internalized, and Modifies Gene Expression: A Novel Approach Of Pre-Programming Stem Cells for Therapeutic Application Requiring Their Chondrogenesis

This appendix contains the manuscript for an experimental study entitled, **"Pentosan polysulfate binds to STRO-1^{+} mesenchymal progenitor cells, is internalized, and modified gene expression: a novel approach of pre-programming stem cells for therapeutic application requiring their chondrogenesis"** published in the journal Stem Cell Research and Therapy. The manuscript describes an investigation of the binding and uptake of pentosan polysulfate by mesenchymal progenitor cells in cell culture and the impact of this on gene expression and proteoglycan biosynthesis.

The candidate, Chris Daly, contributed to concept and revision of the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xx.

RESEARCH

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Pentosan polysulfate binds to STRO-1^{+} mesenchymal progenitor cells, is internalized, and modifies gene expression: a novel approach of pre-programing stem cells for therapeutic application requiring their chondrogenesis

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Abstract

Background: The pharmaceutical agent pentosan polysulfate (PPS) is known to induce proliferation and chondrogenesis of mesenchymal progenitor cells (MPCs) in vitro and in vivo. However, the mechanism(s) of action of PPS in mediating these effects remains unresolved.

In the present report we address this issue by investigating the binding and uptake of PPS by MPCs and monitoring gene expression and proteoglycan biosynthesis before and after the cells had been exposed to limited concentrations of PPS and then re-established in culture in the absence of the drug (MPC priming).

Methods: Immuno-selected STRO-1^{+} mesenchymal progenitor stem cells (MPCs) were prepared from human bone marrow aspirates and established in culture. The kinetics of uptake, shedding, and internalization of PPS by MPCs was determined by monitoring the concentration-dependent loss of PPS media concentrations using an enzyme-linked immunosorbent assay (ELISA) and the uptake of fluorescein isothiocyanate (FITC)-labelled PPS by MPCs. The proliferation of MPCs, following pre-incubation and removal of PPS (priming), was assessed using the Wst-8 assay method, and proteoglycan synthesis was determined by the incorporation of $^{35}SO_4$ into their sulphated glycosaminoglycans. The changes in expression of MPC-related cell surface antigens of non-primed and PPS-primed MPCs from three donors was determined using flow cytometry. RNA sequencing of RNA isolated from non-primed and PPS-primed MPCs from the same donors was undertaken to identify the genes altered by the PPS priming protocol.

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Results: The kinetic studies indicated that, in culture, PPS rapidly binds to MPC surface receptors, followed by internalisation and localization within the nucleus of the cells. Following PPS-priming of MPCs and a further 48 h of culture, both cell proliferation and proteoglycan synthesis were enhanced. Reduced expression of MPC-related cell surface antigen expression was promoted by the PPS priming, and RNA sequencing analysis revealed changes in the expression of 42 genes.

Conclusion: This study has shown that priming of MPCs with low concentrations of PPS enhanced chondrogenesis and MPC proliferation by modifying their characteristic basal gene and protein expression. These findings offer a novel approach to re-programming mesenchymal stem cells for clinical indications which require the repair or regeneration of cartilaginous tissues such as in osteoarthritis and degenerative disc disease.

Keywords: Mesenchymal progenitor cells, Pentosan polysulfate, Heparin, Chondrogenesis, Proliferation, Gene expression, CD146

Background

Adult mesenchymal stem cells (MSCs) are an abundant source of self-renewing, multipotent undifferentiated cells that can be readily isolated from bone marrow, adipose tissue, muscle, and synovium. They can be serially expanded in culture and cryopreserved almost indefinitely without significant loss of their tissue regenerative capacity [1–4]. In-vitro studies have shown that when MSCs are exposed to the appropriate physical, chemical, or biological stimuli they will differentiate into cells of the mesodermal lineage, including osteoblasts, chondrocytes, tenocytes, myocytes, and adipocytes [3–5]. Moreover, when administered systemically, MSCs exhibit the capacity to migrate to the site(s) of tissue injury, where they can modulate inflammatory and immune-regulatory pathways as well as release pro-anabolic factors [6–9]. These unique activities of MSCs have led to extensive investigations into their potential applications as biological agents for the treatment of a variety of clinical applications [5–7]. MSCs have been considered a suitable therapy for muscular skeletal and connective tissue disorders, including degenerative disc disease, osteoarthritis, and repair of articular cartilage, owing to the high incidence of such disorders as well as their limited capacity for spontaneous repair and the limited treatment options [10–16].

As indicated, MSCs possess the ability to localize to sites of tissue injury, suppress inflammation, and facilitate repair. Moreover, there is considerable evidence to suggest that MSCs engraft at these sites, undergo differentiation, and synthesise an extracellular matrix consistent with the endogenous tissue [17, 18]. However, for the regeneration or repair of cartilaginous tissues it is important that the initial differentiation of MSCs to chondrocytes is not followed by further differentiation to osteoblasts, a process that has been observed in some experimental studies using these osteochondral precursors [19, 20].

In previous studies [21] we showed that the incubation of STRO-1⁺ immuno-selected mesenchymal progenitor cells (MPCs) with the pharmaceutical agent pentosan polysulfate (PPS) not only improved their viability and enhanced their chondrogenic differentiation but also suppressed osteogenesis in vitro. In subsequent in-vivo studies using ovine models, MPCs were formulated with PPS and injected directly into degenerate intervertebral discs, and were found to promote the deposition of a new disc matrix without evidence of osteogenic differentiation [22–24]. However, in these animal studies the MPCs and PPS were always mixed together immediately prior to administration. As such, it remained to be determined whether the positive outcomes observed represented the sum of the pharmacological activities of the individual components or whether the mechanism of action was via a reprogramming of MPC genetic expression mediated by PPS.

The objective of the present study was to address this question by examining the concentration-dependent binding and internalization of PPS by MPCs and determine if priming of the cells with the drug changed their genetic signature.

Methods

Preparation of human STRO-1⁺ immuno-selected mesenchymal progenitor stem cells

Bone marrow was collected from the posterior iliac crest of healthy volunteers (20–35 years old) following their informed consent; the procedure was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital (RAH), Adelaide, South Australia. These aspirates were used to prepare immuno-selected STRO-1⁺ MPCs employing procedures described previously [25]. Briefly, STRO-1⁺ mesenchymal precursor cells derived from the bone marrow aspirates were isolated by STRO-1 magnetic activated cell sorting and used to establish primary cultures. The primary cultures were expanded by trypsin-EDTA detachment and re-plating at a density of

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4.0×10^4 cells per cm^2 as previously described [25]. Following 3–4 passages, the cells were harvested by trypsin-EDTA detachment and re-suspended in culture medium at a density of $5.0–20 \times 10^6$ cells/ml. They were then combined with ProFreeze-CDM NAO freezing medium (Lonza Australia Ltd., Blackburn Rd., Mt Waverley, Victoria 3149, Australia) (2×) containing DMSO (7.5%), they were control-rate cryopreserved and placed at −80 °C overnight, and subsequently transferred to the vapour phase of liquid nitrogen until required.

Competitive PPS enzyme-linked immunosorbent assays (ELISAs) of culture media

The concentration of PPS in culture media was determined with a competitive ELISA using a biotinylated monoclonal antibody (1B1) against polysulphated polysaccharides (kindly provided by Professor Prachya Kongtawelert, Department of Biomedical Sciences, Chiang Mei University, Thailand).

Each well of a 96-well plate was coated with 100 μl 50 μg/ml hexadimethrine bromide (Polybrene; Sigma-Aldrich, Sydney, Australia)) in phosphate-buffered saline (PBS), pH 7.4, and incubated at 37 °C for 1 h. The solution was aspirated and the plate was air-dried without washing. Wells were then blocked with 200 μl/well blocking solution (PBS + 1% bovine serum albumin (BSA)) and incubated at 37 °C for 1 h. The solution was aspirated and the wells were washed with 300 μl/well PBST (PBS + 0.05% Tween-20) three times. The plates were flicked to remove the contents of the wells and dried. The monoclonal antibody B1B1 was diluted 1:200 in Dulbecco's modified Eagle's medium (DMEM) and used as the primary antibody solution. The PPS compound (BenePharmachem, Munich, Germany) was used to prepare a 1 mg/ml working stock and was subsequently diluted in DMEM to create a standard curve of 0.004–4 μg/ml. The PPS standard solutions were each mixed with the 1B1 antibody solution in a 1:1 ratio and incubated at 37 °C for 1 h. Aliquots of the inhibition mixtures (100 μl) were transferred to each well and incubated at 37 °C for a further 1 h. Using the same plates, culture media samples containing PPS were mixed 1:1 with the 1B1 antibody solution in the microtitre plate wells. The samples were aspirated from each well and the plate washed with 300 μl/well PBST three times, flicked, and dried. Monoclonal anti-biotin-alkaline phosphatase (AP) antibody (Sigma-Aldrich, Sydney, Australia, cat. no. A-6561) was used as the secondary antibody and was diluted 1:5000 with blocking solution and 100 μl added to each well followed by incubation at 37 °C for 1 h. The antibody solution was aspirated and the plate was washed with 300 μl/well PBST three times, flicked, and dried. The AP substrate, para-nitrophenyl phosphate (PNP; 200 μl 1 mg/ml PNP in 0.1 M $NaHCO_3$ buffer containing 2 mM $MgCl_2$, pH 8.6) was added to each well and the plates incubated in the dark for 20 min. Absorbance at 405 nm was then determined with a micro-plate reader. All assays were performed in triplicate.

Kinetics of PPS uptake by MPCs in culture

Primary MPC monolayers were established in culture as described previously [21]. Briefly, 3.0×10^5 MPCs were seeded into wells of 48-well plates and incubated with DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 16 h. The media from the primary cultures was discarded and the wells were washed with DMEM (3×500 μl/well); media and washings were discarded and then replaced with DMEM (500 μl/well) containing gradient concentrations of PPS (0.5, 1.0, 2.5, 5, and 10 μg/ml/well). Plates were maintained at 37 °C in 95% air/CO_2 and, after 0.25, 0.50, 2, 6, 20, and 24 h, media from individual wells were aspirated, cells washed (PBS, 0.5 ml/well) and media and washings pooled. The concentrations of PPS remaining in the aspirated media and washings of the cultures at each time point was determined using the PPS ELISA as described above.

The preparation of fluorescein isothiocyanate (FITC)-labelled PPS

PPS (100 mg) was converted to the tetrabutyl ammonium (TBA) salt by incubating with tetrabutylammonium bromide (100 mg; Sigma-Aldrich, Sydney, Australia) dissolved in 10 ml de-ionized H_2O for 4 h at ambient temperature. The PPS-TBA complex was dialyzed against de-ionized water for 24 h to remove excess salts and then lyophilized. The PPS-TBA complex (50 mg, dissolved in 1 ml DMSO) was mixed with 1,1-carbonyl di-imidazole (28.0 mg/0.5 ml; Sigma-Aldrich, Sydney, Australia)) and incubated at 56 °C for 1 h. After cooling to room temperature, hydrazine (47.8 mg; Sigma-Aldrich, Sydney, Australia) was added and the solution incubated with shaking for 16 h at 45 °C. The PPS carboxyhydrazide complex was then reacted with FITC (Sigma-Aldrich, Sydney, Australia) using the manufacturer's instructions to convert the FITC-PPS derivative into a TBA salt derivative. The PPS-FITC-TBA salt was then converted to the sodium salt by mixing at 4 °C with 4.0 M NaCl (100 ml) for 16 h followed by 48 h dialysis against water with changes every 16 h, and then lyophilized. The lyophilized PPS-FITC derivative was purified by size-exclusion chromatography on a Superdex-200 column (GE Healthcare Ltd., Sydney, Australia) equilibrated in 0.25 molar NaCl. Column fractions were monitored for PPS concentration using the dimethyl-methylene blue assay [26] and FITC by fluorescence excitation/emission at 485/538 nm. Fractions positive for PPS and FITC fluorescence were pooled, desalted, and lyophilized. The purity of the PPS-FITC complex was

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established by NMR spectroscopy (by Dr. Ronald Shimmon, Department of Chemistry, University of Technology, Sydney, Australia).

PPS-FITC uptake by MPCs using fluorescence microscopy
Fluorometric assay

Primary MPC monolayer cultures were established in six-well plates (2.5×10^5 cells/well) as described previously [21]. After 16 h, DMEM (3 ml) containing various concentrations of PPS-FITC (0, 1.0, 2.5, 5.0, 10.0, and 20.0 μg/ml) were added to the wells and incubated at 37 °C in 5% CO_2 for a further 24 h. The media were collected from each well, and the cells were washed 3× with PBS at room temperature. Media and washings were discarded. The washed cells were released from the plates with 250 μl 0.25% trypsin/EDTA at 37 °C for 10 min. The cells and supernatant were separated by centrifugation at 500 g for 10 min, the supernatants were discarded, and the cell pellets washed 3× with PBS (1 ml/well). The cell pellets derived from each culture well were re-suspended in 100 μl de-ionised H_2O then transferred to wells of black microplates. The microplates were agitated for 1 h to lyse the cells in the absence of light, and the intensity of the fluorescence emission at 538 nm determined for all added PPS-FITC concentrations using a fluorescence microplate reader (Labsystems Fluoroskan II, ThermoFisher Scientific Australia Pty. Ltd., Scoreby, Australia) with de-ionised H_2O as a blank. The levels of PPS-FITC in each well were quantified using a standard curve prepared from the purified PPS-FITC prepared above.

Qualitative assay

MPCs (6000 cells/well) were seeded on eight-well slides (Lab-Tek-II® Chamber Slide System, Permanox®, Grand Island, NY, USA) and incubated at 37 °C in a 5% CO_2 atmosphere for 16 h. DMEM media (1.0 ml) containing 0, 0.5, 1.0, and 2.5 μg/ml PPS-FITC was added to each well and the slides incubated for a further 24 h at 37 °C. The media were removed and the bound cells washed 3× with 1.0 ml PBS. Media and washings were discarded and cells fixed using 300 μg/well HistoChoice MB Fixatives (Amresco, Solon, OH, USA) for 20 min at room temperature. After washing once with PBS, the cells of each slide were stained with 20 μg/ml propidium iodide (PI) for 10 min at room temperature, washed 3× with PBS, once with 70% ethanol, and 3× with absolute ethanol, and then viewed under UV light using a Nikon Eclipse 80 fluorescence microscopic (Coherent Scientific, Hilton, Australia). Cells were viewed for FITC and PI fluorescence using excitation and emission wavelengths of 485/538 nm for 2 s and 535/620 nm for 60 s, respectively. The cell images were captured using a digital camera coupled to the microscope and images analysed using the NIS-Elements software (Coherent Scientific, Hilton, Australia).

Assessment of MPC proliferation alone and after priming with PPS

Triplicate cultures of passage 4 MPCs at densities of 1×10^6 cells/ml were established in 24-well plates as described previously [21]. High-glucose DMEM containing 5 μg/ml PPS was then added to 12 wells of the plates and an equivalent volume DMEM alone to the remaining wells. After incubation for 24 h, media were removed from all wells and cells were washed 3× with PBS and then re-established in culture. After 4, 24, and 48 h, incubations were stopped, media removed and cells washed 3× with PBS; media and washings were then discarded. Cells were released from the plates by trypsin/EDTA treatment, the harvested cells from each well were re-suspended in PBS, and aliquots were then analysed to determine MPC proliferation for each of the culture time periods using a commercial cell counting kit (Wst-8 Kit (CCK-8); Sigma-Aldrich, Sydney, Australia) according to the manufacturer's instructions. As the non-PPS primed MPC cultures failed to demonstrate significant variation in their proliferation over the three time periods, the values obtained from each incubation period were pooled and used as the non-PPS pre-treatment control.

Proteoglycan synthesis by MPCs alone and after priming with PPS

Wells of six-well culture plates were seeded with passage 4 MPCs (2.8×10^5/well) and incubated with DMEM + 10% FBS at 37 °C in 5% CO_2 for 16 h. High-glucose DMEM containing 5 μg/ml PPS was then added to three wells of the plates and DMEM alone to the remaining three wells. After incubation for 24 h, media were removed from all wells and cells were washed twice with PBS (3 ml/well) and then re-established in culture. The biosynthesis of proteoglycans (PGs) by these cells over 24 h was then determined as previously described [21]. Briefly, media (3 ml) containing 2.2 μCi/ml $H_2^{35}SO_4$ (Perkin-Elmer Life and Analytical Science Knoxfield, Victoria, Australia) was added to each well and plates incubated for 48 h. The medium was removed and discarded. Cells were washed with 3× PBS, and then collagenase solution (Sigma Aldrich, Sydney, Australia; 500 μl, 1 mg/ml) was added to each well and the plate incubated at 37 °C for 1 h to detach the cells and matrix from the plates. The collagenase digests were transferred to 1.5-ml tubes and an equal volume of acetate-buffered papain (Sigma-Aldrich, Sydney, Australia; 1 mg/ml) added to each tube. After incubation at 65 °C for 1.5 h, aliquots (100 μl) of the digests were assayed

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for DNA content [27] and the remainder transferred to 1.5-ml tubes, and 40 μl 1 mg/ml chondroitin sulphate A (Sigma Aldrich, Sydney, Australia) and 60 μl 5% aqueous cetyl pyridinium chloride (CPC; Sigma Aldrich, Sydney, Australia) was added. The tubes were vortexed and then centrifuged at 11,000 rpm for 3 min to pellet the precipitated ^{35}S-glycosaminoglycan (GAG)-CPC complex. The precipitates were collected by centrifugation, washed (3× PBS), and then dissolved in 1 ml scintillant (Perkin-Elmer Life and Analytical Science Knoxfield, Victoria, Australia) and transferred to a scintillation vial. The radioactivity of ^{35}S incorporated to newly synthetized S-GAGs of the PGs was determined by scintillation counting (Perkin-Elmer Tricarb 2910TR, Perkin-Elmer Corp., Massachusetts, USA). Results were calculated as ^{35}S-GAG-DPM/μg DNA as an index of proteoglycan synthesis per cell.

Monitoring of MPC phenotypic receptors by flow cytometry
Suspensions of passage 4 MPCs (2.5×10^5) derived from three independent healthy young donors (RAH1, RAH2, and RAH3) were seeded into each well of a six-well plate (in duplicate) and incubated with DMEM + 10% FBS at 37 °C in 5% CO2 for 16 h. The next day, DMEM containing 5 μg/ml PPS was added to three wells of both six-well plates. The remaining three wells of the same plates only received DMEM and were used as the controls (MPCs alone). After an additional 24 h, the cultures from one plate were terminated. The remaining plate was incubated for a further 24 h (i.e. a total incubation time of 48 h). At termination, all media were removed and the six wells of the plates were washed twice with PBS (3 ml/well). Media and washings were discarded, and MPCs were detached from wells by trypsin/EDTA treatment; enzyme activity was quenched and the cells were strained through a 70-μm cell strainer (Becton Dickinson Biosciences, CA, USA) to ensure preparation of single cell suspensions. The MPC suspensions were washed with 10 ml wash buffer (Hank's buffered salt solution + 5% fetal calf serum (FCS)) and then centrifuged at 400 *g* for 7 min at 4 °C. Cells were re-suspended in blocking buffer (wash buffer supplemented with 1% (v/v) normal human serum + 1% v/v BSA) and counted in 0.4% Trypan Blue and left on ice in blocking buffer for 30 min. Cells were then pelleted by centrifugation (400 g for 7 min at 4 °C), and the supernatant removed and discarded. The cell pellet was re-suspended in 100 μl of one of the primary antibody listed in Table 1 at a final concentration of 20 μg/ml per tube or 100 μl neat supernatant antibody. After maintaining the tubes at 4 °C for 45–60 min, cells were washed twice with 2 ml cold wash buffer and centrifuged at 400 g

Table 1 Primary and secondary antibodies used for MPC ± PPS cytometric analysis

Primary antibodies	Type	Origin
Stro-1	In-house antibody	Provided by Prof. S. Gronthos and Prof. A. Zannettino
CD73	Purified mouse anti-human CD73	BD Pharmingen 550256
CD90	Biotin mouse anti-human CD90	BD Pharmingen 555594
CD105	Purified mouse anti-human CD105	BD Pharmingen 555690
CD44 (H9H11)	In-house antibody	Provided by Prof. S. Gronthos and Prof A. Zannettino
CD146 (CC9)	In-house antibody	
CD34	CD34 FITC	Beckman Coulter IM1870
CD45	CD45 FITC	Beckman Coulter IM0782U
CD14	CD14 FITC	Beckman Coulter IM0645U
Secondary antibodies		
Streptavidin FITC conjugate		Invitrogen SA1001
IgM FITC	Goat anti-mouse IgM FITC	Southern Biotech 1020-02
IgG FITC	Goat anti-mouse IgG FITC	Southern Biotech 1030-02
Negative Controls		
IgM	1A6.12 isotype-matched negative control/ anti-salmonella	Provided by Dr. L Ashman
IgG1	1B5 isotype-matched negative control/ anti-salmonella	Provided by Dr. L Ashman
IgG2a	1D4.5 isotype-matched negative control/ anti-salmonella	Provided by Dr. L Ashman

CD cluster differentiation, *FITC* fluorescein isothiocyanate, *Ig* immunoglobulin

for 7 min at 4 °C. Cells were re-suspended in 100 μl blocking buffer containing the appropriate secondary goat anti-mouse antibody or FITC-conjugated antibody at a 1:50 dilution (Southern Biotechnology, USA) (Table 1) and incubated for 30 min and then washed twice with 2 ml cold wash buffer at 400 g for 5 min at 4 °C. Antibody-labelled MPCs were then re-suspended in 0.5 ml FACS FIX (1% (v/v) formalin, 0.1 M D-glucose, 0.02% sodium azide, in PBS) for flow cytometric analysis using a BD FACS Canto II and Flow Data Analysis Software V10 (Becton Dickinson Biosciences, CA, USA).

Extraction of RNA from MPC cultures and genomics analysis
Cells from the three donors (RH1, RH2, and RH3) were used for these studies. Each cell line was processed as described above for flow cytometric analysis but cells

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were detached from plates using TrypLE select (Gibco 12563-029), an animal origin-free cell dissociation reagent, which was then inactivated by diluting with Hanks buffer without FCS. Cells were pelleted by centrifugation at 400 g for 7 min at 4 °C, and the supernatant removed. Cells were re-suspended and washed again with Hanks buffer then lysed using 700 μl QIAzol (Qiagen #79306). The RNA was isolated using a MiR-Neasy Mini Kit (Qiagen #217004) and the on-column DNAse treatment was performed according to the manufacturer's instructions (RNAse free DNase set; Qiagen #79254). RNA concentrations were measured using a Nanodrop reader. The RNA samples were processed by automated RNASeq-FastQ sequencing using the NEXTflex™ Rapid Illumina Directional RNA-Sequencer (BIOO Scientific, Austin, Texas, USA); for each sample, 300 ng of total RNA was processed using the NEXTflex™ Rapid Illumina Directional RNA-Seq Library Prep Kit (BIOO Scientific, Austin, Texas, USA). Briefly, the method selects poly-adenylated mRNA with coated beads and then converts them to strand-preserved cDNA (via dUTP) before the ligation of sequencing adapters and barcodes. After PCR amplification for 15 cycles the samples were quantified by a fluorescence assay before pooling in equimolar ratios for sequencing. The sample pool was sequenced by the Illumina Nextseq 500 sequencer using a High Output v2 (2×75 bp) paired-end sequencing kit ((Illumina, San Diego, USA)) as per the manufacturer's instructions except that the loading concentrations were reduced by 30% to 0.9 pM. The data were analysed with de-multiplexed reads that were aligned (human hg38) using the TopHat aligner and the differential expression of transcripts was assessed using Cufflinks in Illumina's Base-space analysis cloud.

Statistical methods

All data analysis and graphical representations were performed using Microsoft Excel for Mac (Microsoft version 15.33) and Prism for Mac (version 7.0b, GraphPad Software Inc.). Parametric data were analysed using one-way analysis of variance (ANOVA), with Tukey's multiple comparison test undertaken when significant differences in means were observed. Non-parametric data were analysed using the Kruskal-Wallis test of median values followed by Dunn's multiple comparison test. Treated/non-treated groups were compared using the two-tailed Student's t test followed by Mann-Whitney U tests. P values < 0.05 were considered statistically significant. For the genomic cDNA sequencing, analysis of statistical differences in gene levels in cells from the 24- and 48-h primed and non-primed MPC cultures were determined using the manufacturers' software with q values < 0.045 being accepted as
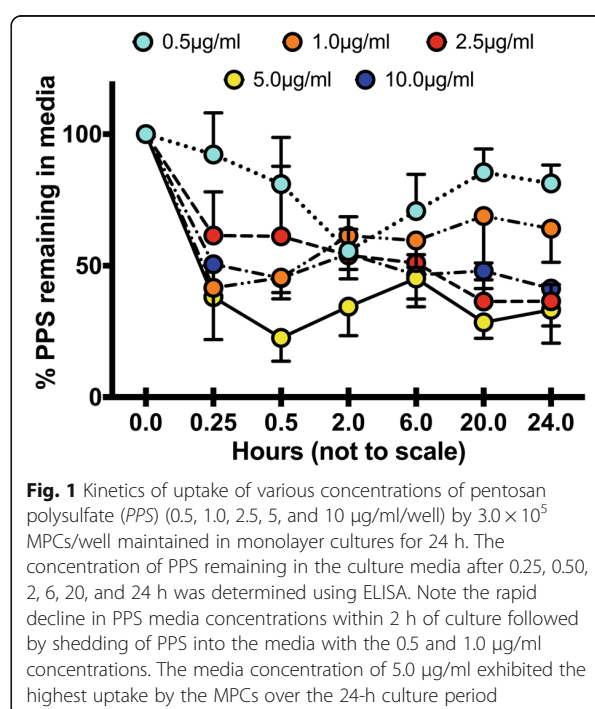

Fig. 1 Kinetics of uptake of various concentrations of pentosan polysulfate (*PPS*) (0.5, 1.0, 2.5, 5, and 10 μg/ml/well) by 3.0×10^5 MPCs/well maintained in monolayer cultures for 24 h. The concentration of PPS remaining in the culture media after 0.25, 0.50, 2, 6, 20, and 24 h was determined using ELISA. Note the rapid decline in PPS media concentrations within 2 h of culture followed by shedding of PPS into the media with the 0.5 and 1.0 μg/ml concentrations. The media concentration of 5.0 μg/ml exhibited the highest uptake by the MPCs over the 24-h culture period
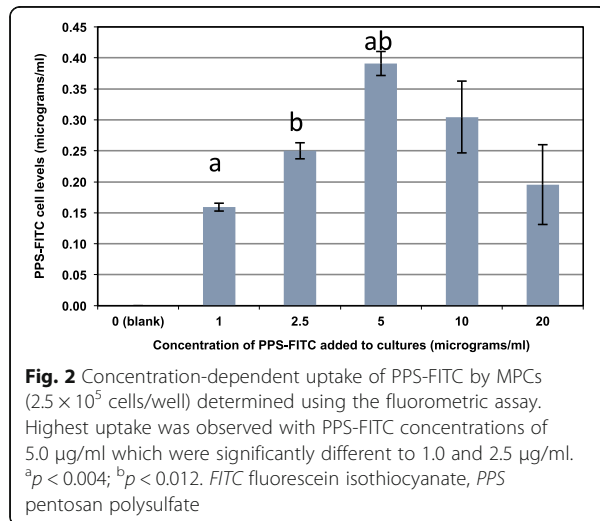
significant. However, for the majority of gene changes identified, statistical significance was observed at the $q = 0.017$ level.

Results

Kinetics of binding and uptake of PPS by MPCs in culture

The kinetics of binding and uptake of PPS by cultured MPCs when added to the media at concentrations of 0.5–10 μg/ml was monitored by the percentage decrease in their media levels over 24 h using the PPS ELISA. As shown in Fig. 1, all concentrations of PPS added to the culture media decreased over the first 0.5–2.0 h of incubation with MPCs. For media concentrations of 0.5 and 1.0 μg/ml PPS, this initial decline was followed by a partial release of PPS into the media over the subsequent 6–24 h (shedding period). However, for cultures spiked with 2.5, 5.0, or 10.0 μg/ml PPS, the reduced media levels were sustained over this period. Interestingly, cultures to which 5.0 μg/ml PPS had been added demonstrated the highest decline in media levels after 0.5 h and only released relatively small amounts over the subsequent 24-h period (Fig. 1). These observations suggest a rapid binding of PPS to cell surface heparin receptors, followed by a time- and concentration-dependent shedding and uptake by the MPCs over the 24 h of culture [28, 29]. Moreover, under the conditions used for these cultures, optimum uptake of PPS by MPCs was found to occur with a medium concentration of 5.0 μg/ml.

As the PPS ELISA was not sufficiently sensitive to evaluate the amounts of PPS associated with the MPCs

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Fig. 2 Concentration-dependent uptake of PPS-FITC by MPCs (2.5×10^5 cells/well) determined using the fluorometric assay. Highest uptake was observed with PPS-FITC concentrations of 5.0 μg/ml which were significantly different to 1.0 and 2.5 μg/ml. [a]$p < 0.004$; [b]$p < 0.012$. *FITC* fluorescein isothiocyanate, *PPS* pentosan polysulfate
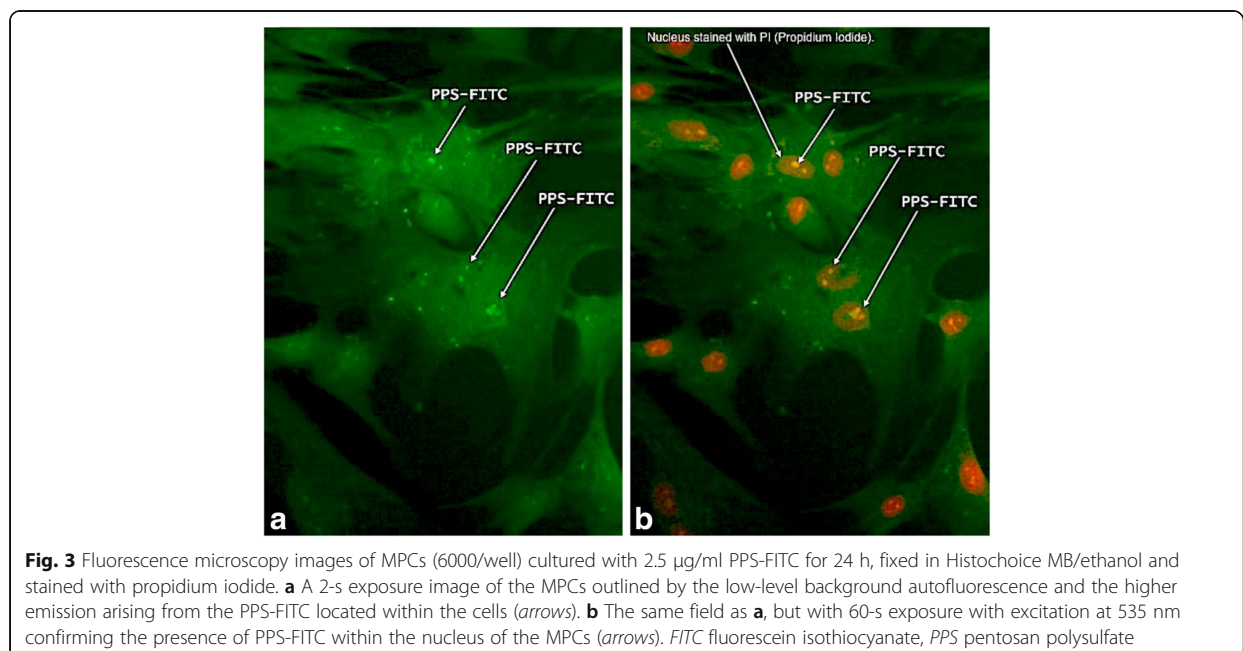
following their removal from culture, we used the PPS-FITC preparation and a fluorometric assay to assess the amounts of PPS associated with the MPCs. This was coupled with fluorescence microscopy to identify the intra-cellular distribution of PPS over the indicated time points. The results of these studies are shown in Figs. 2 and 3. As is evident from Fig. 2, significantly higher levels of PPS-FITC were associated with the MPCs after 24 h of culture with 5.0 μg/ml than with 1.0 μg/ml ($p < 0.004$), 2.5 μg/ml ($p < 0.012$), or 20 μg/ml as a trend ($p < 0.054$). However, significant difference could not be demonstrated between media concentration of 5.0 and 10.0 μg/ml using the PPS-FITC fluorometric assay.
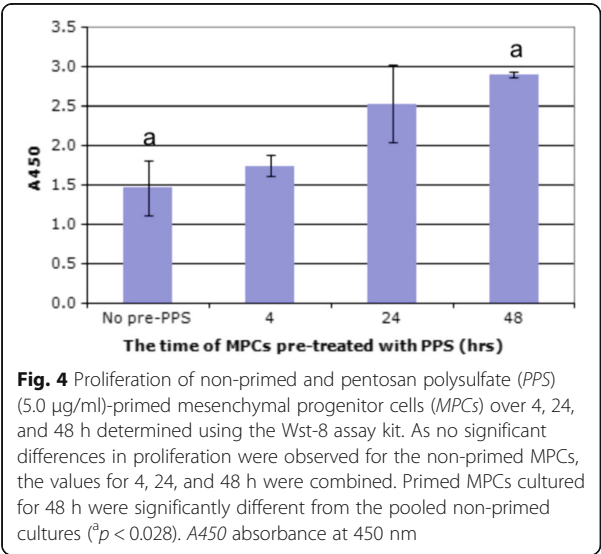
Qualitative studies of the interaction of PPS-FITC with MPCs using fluorescence microscopy together with co-staining of the preparations with the selective nucleus stain PI showed that, after 16 h of culture, the PPS-FITC was largely located within the nucleus of the cell (Fig. 3).

Although the kinetic and fluorometric studies on the uptake of PPS by MPCs suggested that with media concentrations of 5.0 μg/ml more than 50% of the agent was bound and internalised by the cells, the culture periods used never exceeded 24 h. A study was therefore undertaken to monitor MPC proliferation when the cells were cultured alone or after pre-incubation (priming) with 5.0 μg/ml PPS for 4, 24, and 48 h. The results of this study are shown in Fig. 4 where it is evident that MPCs primed with PPS increased proliferation after 48 h to a significantly higher extent than non-primed MPCs ($p < 0.028$). As an earlier study [21] had reported that co-cultures of MPCs with PPS promoted chondrogenic differentiation, we next investigated the biosynthesis of PGs of MPCs alone and after pre-culturing with PPS as described for the proliferation study.
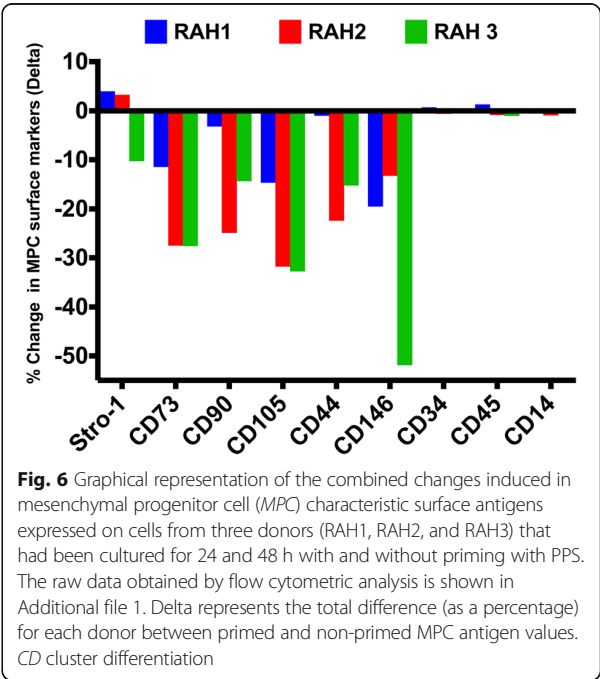
The results of this experiment are shown in Fig. 5 and demonstrate that the MPCs primed with PPS increased de novo PG biosynthesis to a greater extent than when MPCs were cultured alone ($p < 0.005$). Since the PPS priming process was known to promote MPC proliferation (Fig. 4), we normalized the incorporation of $^{35}SO_4$

Fig. 3 Fluorescence microscopy images of MPCs (6000/well) cultured with 2.5 μg/ml PPS-FITC for 24 h, fixed in Histochoice MB/ethanol and stained with propidium iodide. **a** A 2-s exposure image of the MPCs outlined by the low-level background autofluorescence and the higher emission arising from the PPS-FITC located within the cells (*arrows*). **b** The same field as **a**, but with 60-s exposure with excitation at 535 nm confirming the presence of PPS-FITC within the nucleus of the MPCs (*arrows*). *FITC* fluorescein isothiocyanate, *PPS* pentosan polysulfate

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Fig. 4 Proliferation of non-primed and pentosan polysulfate (*PPS*) (5.0 μg/ml)-primed mesenchymal progenitor cells (*MPCs*) over 4, 24, and 48 h determined using the Wst-8 assay kit. As no significant differences in proliferation were observed for the non-primed MPCs, the values for 4, 24, and 48 h were combined. Primed MPCs cultured for 48 h were significantly different from the pooled non-primed cultures ([a]$p < 0.028$). *A450* absorbance at 450 nm

Fig. 6 Graphical representation of the combined changes induced in mesenchymal progenitor cell (*MPC*) characteristic surface antigens expressed on cells from three donors (RAH1, RAH2, and RAH3) that had been cultured for 24 and 48 h with and without priming with PPS. The raw data obtained by flow cytometric analysis is shown in Additional file 1. Delta represents the total difference (as a percentage) for each donor between primed and non-primed MPC antigen values. *CD* cluster differentiation

into the S-GAGs of the newly synthetized PG relative to cell numbers (DNA content).

In view of these findings, we next sought to determine, using flow cytometry, if the PPS priming process also induced changes in the MPC cell surface phenotypic antigens after culturing the primed and non-primed cells for 24 and 48 h. The results of these studies are shown in Fig. 6 and Additional file 1, where the net differences between primed and non-primed MPC antigen levels were calculated for each donor and expressed as their delta change. Figure 6 depicts graphically the total delta changes that occurred in surface antigen levels for each donor over the 24- and 48-h culture periods. As is shown, donors RAH2 and RAH3 exhibited patterns of changes with marked decreases in the CD73, CD90, CD105, and CD44 surface antigens of between 15–30%. However, expression of CD146 on MPCs from donor

Fig. 5 Biosynthesis of PGs determined by the incorporation of $^{35}SO_4$ into their sulphated glycosaminoglycans (*35S-GAG*) in 24-h cultures of non-primed MPCs and MPCs primed with pentosan polysulfate (*PPS*). Primed MPCs synthesised 40% more PGs than non-primed MPCs after the 24-h culture. [a]$p < 0.005$

RAH3 declined by more than 50%. MPCs from donor RAH1 were found to be less responsive to the priming procedure but still exhibited the same pattern of decline in the characteristic MPC surface phenotype receptors. Interestingly, the STRO-1 marker used to isolate the MPCs from bone marrow aspirates was not markedly affected by the priming step; only donor RAH3 exhibited a 10% decrease, with the cells from the other two donors showing minimal change in expression of this antigen following the PPS priming procedure. The low levels of the hematopoietic and monocyte cell markers CD34, CD45, and CD14 were not affected by PPS priming, suggesting preservation of the mesenchymal cell lineage (Fig. 6).

Additional evidence to support the finding that priming of MPCs with PPS mediated altered gene expression by these cells was provided by isolating the RNA extracted from MPCs of the three donors after culturing for 24 and 48 h and undertaking RNASeq-FastQ sequencing. The results of this study are shown in Tables 2 and 3, which record the mean statistically significant gene changes for the three donors that were detected between their primed and non-primed MPCs after 24 and 48 h of culture. Using internet-based gene search engines, the proteins encoded by these genes are also identified in Tables 2 and 3. These datasets show that after the initial 24 h of culture only four genes were upregulated and 16 downregulated (Table 2) by the priming process. However, after 48 h 16/42 genes were upregulated and 26 downregulated.
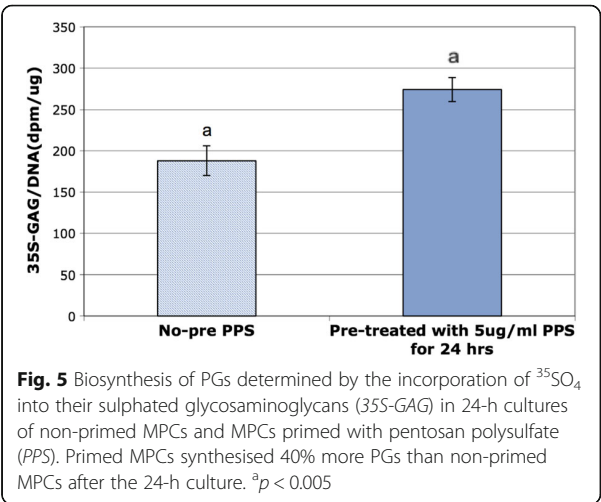
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Table 2 Gene expression changes induced by 24-h cultures of MPCs with 5.0 μg/ml PPS relative to identical cultures of non-primed MPCs

Gene	Fold change	Regulation	Primary functions[a]
ACTA2*	1.21	Down	Encoding actin-2 protein a member of the actin family which collectively are responsible for cell motility, structure and integrity.
ADAMTSL4	0.77	Down	Encodes the protein ADAMTS4 which lacks a C-terminal TS motif but when proteolytically processed generates the mature proteinase that degrades aggrecan, a major component of hyaline cartilage.
ANK1*	2.79	Up	Encoding the protein Ankyrin-1 a member of the Ankyrin family that play key roles in cell motility, activation, and proliferation.
COL11A1*	1.21	Down	Encoding one of the alpha chains of type XI collagen.
COL5A3*	2.22	Up	Encodes one of the alpha chains of type V collagen
COMP*	1.08	Down	COMP gene provides the instructions for making the COMP protein, an important regulatory component of the extracellular matrix.
DACT1	1.28	Down	Encodes a protein member of the Dapper family. It interacts with and positively regulates dishevelled-mediated signalling pathways during development and is an antagonist of beta-caterin.
ENPP1	0.58	Down	Encoded protein is type II transmembrane glycoprotein that cleaves a variety of substrates including phosphodiester bonds of nucleotides.
FLG*	1.82	Down	The FLG gene provides instructions for making the large protein profilaggrin.
GREM2	0.64	Down	Encodes a member of the BMP antagonist family likely by binding to BMPs
HSPB7*	1.56	Down	Encodes a member of the heat shock beta-7 protein family
LARGE	0.94	Down	Encodes members of the N-acetylglucosamine-L-transferase protein family responsible for glycosylation of glycoproteins and glycosphingolipids.
LMOD1	0.52	Down	Encodes the leicmodin 1 protein that has a putative membrane-spanning region and two types of tandemly repeat blocks.

Table 2 Gene expression changes induced by 24-h cultures of MPCs with 5.0 μg/ml PPS relative to identical cultures of non-primed MPCs *(Continued)*

Gene	Fold change	Regulation	Primary functions[a]
LOXL4	0.64	Down	Encodes a member of the lysyl oxidase family essential for the biogenesis of crosslinks of matrix collagens and elastins.
LRRC15	0.76	Down	Encodes the leucine rich repeat containing 15 protein that constitute regions of the small proteoglycans.
MRVI1	0.83	Down	Encoding protein MRVI1, a substrate of cGMP-dependent kinase-1(PKG1).
SCUBE3	0.86	Down	Encodes Signal peptide-CUB and EGF-like Domain-containing Protein3.
SVIL	0.64	Up	Encodes the protein Supervillin which is tightly associated with actin filaments and plasma membranes.
SYNPO2	1.06	Down	Encodes Synaptodin 2-like protein, GO annotations include actin binding.
TM4SF1	0.72	Up	Encodes a member of the transmembrane 4 superfamily that mediate signal transduction in the regulation of development, activation, and growth.

[a] From Gene Cards Human Gene Database Index, Weizmann Institute of Science, 234 Herzi Street, Rehovat 7610001, Israel
* Confirmed via alternative analysis (Star/DESeq)
ADAMTS a disintegrin-like metalloproteinase with thrombospondin motifs, *COMP* cartilage oligomeric matrix protein, *GO* gene ontology, *MPC* mesenchymal progenitor cell, *PPS* pentosane polysulfate

Discussion

This study has shown that priming of MPCs with PPS results in the initial binding of the drug to the cell surface receptors accompanied by partial shedding, and then internalization and migration to the cell nucleus where it influenced gene and protein expression. The extent of changes induced in MPC cell surface markers by the PPS priming step for the three donors was found to be variable (Fig. 6). Indeed, differences in gene expression by bone marrow-derived MSCs from different donors have been previously reported as a potential problem for their routine application in clinical practice [30]. This inter-donor variability has also been attributed to a variety of other factors, including the inherent heterogeneity of the MSC populations isolated from different individuals, the duration of their culture expansion, and the period and nature of their storage [31–33]. The MPCs used in the present study were all within the age range of 20–35 years, were selected on the basis of their expression of STRO-1, and were subjected to similar culture and storage conditions to minimize inter-donor cell variability.

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Table 3 Gene expression changes induced by 48-h cultures of MPCs with 5.0 μg/ml PPS relative to identical cultures of non-primed MPCs

Gene	Fold change	Regulation	Primary functions[a]
ABCA8	2.6	Up	The ABCC8 gene provides instructions for making the sulfonylurea receptor 1 (SUR1) protein. The SUR1is a subunit of the ATP-sensitive potassium (K-ATP) channel.
ABI3BP	0.9	Up	Encodes the ABI family member 3 (NESH) binding protein. GO annotations of this gene include heparin and collagen binding.
ACAN	0.8	Up	Encoding for the Aggrecan core protein, also known as cartilage-specific proteoglycan core protein (CSPCP) or chondroitin sulfate proteoglycan 1.
ASNS*	1.1	Down	The ASNS gene encodes the enzyme asparagine synthetase (EC 6.3.5.4)
CACNA2D1	1.1	Up	Encoding calcium voltage-gated channel auxiliary subunit alpha2delta 1 that mediates calcium channel regulatory activity.
CBS	1.0	Down	Cystathionine β-synthase (CBS; l-serine hydro-lyase) adding homocysteine homocystinuria.
CD74	2.0	Up	HLA class II histocompatibility antigen gamma chain also known as HLA-DR antigens-associated invariant chain or CD74.
CHI3L1	1.5	Up	Chitinase-3-like protein 1, also known as YKL-40, is a secreted glycoprotein.
CNN1	0.7	Down	Encodes a matricellular protein also known as epididymis protein 1 that induces fibroblast senescence and has been reported to restrict fibrosis in cutaneous wound healing.
COMP	3.2	Down	COMP gene provides the instructions for making the COMP protein, an important regulatory component of the extracellular matrix.
CRISPLD2	0.8	Down	Cysteine rich secretary protein LCCL domain 2, exhibits significant LPS binding affinity.
DDIT4	0.7	Down	DNA damage inducible transcript 4 regulates cell growth, proliferation and survival via inhibition of the activity of the mammalian target of rapamycin complex 1 (mTORC1).
FLG	1.2	Down	The FLG gene provides instructions for making the large protein profilaggrin
FOSB	101.0[#]	up	FosB transgene is associated with the induction of the AP-1 complex. FosB interacts with Jun oncoproteins enhancing their DNA binding activity.
GGT5	1.8	Up	Encodes the gamma-glutamyl-transpeptidase protein family. After post-translational modification, the protein can convert Leukotriene C4 to Leukotriene D4.
FST	0.7	Up	Encodes Follistatin, also known as activin-binding protein. Its primary function is the binding and bioneutralization of members of the TGF-β superfamily.
GHRL	84.6[#]	Down	Encodes Growth Hormone protein releasing peptides protein.
HIST2H3A	99.4[#]	Up	Encodes Histone Cluster 2, H3a protein. Histones play a central role in transcription regulation, DNA repair, and regulation of gene expression.
HMGA1	0.6	Up	Encodes High Mobility Group AT-Hook 1 that regulates inducible gene transcription.
HMGA2	0.8	Up	Encodes High Mobility Group AT-Hook 2, a protein coding gene which contains structural DNA binding regions that may act as transcriptional regulating factors.
IGF2	1.6	Up	Encodes the Insulin-Like Growth Factor 2 protein family that play essential roles in growth and development.
LARGE	0.8	Down	Encodes members of the N-acetylglucosaminyltransferase protein family responsible for glycosylation of glycoproteins and glycosphingolipids.
LRRC15	1.8	Down	Gene encoding Leucine Rich Repeat Containing 15 Proteins. GO annotations related to this gene include collagen binding and laminin binding.
MASP1	1.9	Down	Gene encoding mannan binding lectin serine peptidase 1 that regulates the lectin pathway of complement activation.
METTL7A	1.4	Up	Encodes Methyltransferase Like 7A protein. GO annotations related to this gene include methyltransferase activity and S-adenosylmethionine-dependent methyltransferase activities.
MTHFD2*	0.9	Down	Encodes methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2 enzyme, activities that allows binding of NAD.
NFATC2	1.8	Down	Encodes for Nuclear factor of activated T-Cells 2 protein that resides in the cytosol and only translocate to the nucleus upon T-cell receptor stimulation where it becomes a member of the nuclear factors of the activated T-cell transcriptional complex.
OLFML2A	1.7	Up	Encodes for Olfactomedin-Like 2A protein. GO annotations related to this gene include protein homodimerization activity and extracellular matrix binding.
PAMR1	1.2	Up	Encoding peptidase domain containing associated with the muscle regeneration 1

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Table 3 Gene expression changes induced by 48-h cultures of MPCs with 5.0 μg/ml PPS relative to identical cultures of non-primed MPCs *(Continued)*

Gene	Fold change	Regulation	Primary functions[a]
PHGDH*	1.1	Down	Encoding D-3-phosphoglycerate dehydrogenase (catalyses the transition of 3-phosphoglycerate into 3-phosphohydroxypyruvate, which is the committed step in the phosphorylated pathway of L-serine biosynthesis. It is also essential in cysteine and glycine biosynthesis.
PIM1	0.8	Down	Encoding Proto-oncogene serine/threonine-protein kinase Pim-1. It plays a role in signal transduction in blood cells, contributing to cell proliferation and survival.
POM121L9P	2.9	Up	This gene encodes a transmembrane protein that localizes to the inner nuclear membrane and forms a core component of the nuclear pore complex, which mediates transport to and from the nucleus.
PSAT1	1.8	Down	Encoding spermidine/spermidine Ni-acetyltransferase 1 which is a rate limiting enzyme in the catabolic pathway of polyamine metabolism.
PTX3	1.2	Up	Encoding pentraxin-related protein PTX3 also known as TNF-alfa induced protein 5. The expression of this protein is induced by inflammatory cytokines in response to inflammatory stimuli in several mesenchymal and epithelial cell types. It also plays a role in angiogenesis and tissue remodelling.
SLC38A1*	0.9	Down	Encoding sodium-coupled neutral amino acid transporter 1, production of which plays an essential role in the uptake of nutrients, energy production, chemical metabolism, and detoxification.
SLC7A11	1.3	Down	Encoding solute carrier family 7 member 11 protein that is highly specific for cystein and glutamate amino acids.
SLC7A5	0.8	Up	Encoding solute carrier family 7 member 5 protein that transports large neutral amino acids.
SVIL	0.7	Up	Encodes Supervillin. The gene product is tightly associated with both actin filaments and plasma membranes, suggesting a role as a high-affinity link between the actin membranes, suggesting a role as a high-affinity link between the actin and the membrane.
THSD4	0.5	Up	Encoding thrombospondin type-1 domain containing protein 4. The thrombospondin family members are adhesive glycoproteins that mediate cell-to-cell and cell-to-matrix interactions.
TMEM200A	0.8	Down	Encoding transmembrane protein 200A
TPPP3	122.5[#]	Down	Tubulin polymerisation promoting protein family member 3 a protein encoding gene. GO annotations of this gene include tubulin binding.

[a]From Gene Cards Human Gene Database Index, Weizmann Institute of Science, 234 Herzi Street, Rehovat 7610001, Israel
*Confirmed via alternative analysis (Star/DESeq)
[#]Fold-change overestimated due to 'zero' measurement in one sample
ADAMTS a disintegrin-like metalloproteinase with thrombospondin motifs, *COMP* cartilage oligomeric matrix protein, *GO* gene ontology, *MPC* mesenchymal progenitor cell, *LPS* lipopolysaccharide, *PPS* pentosane polysulfate, *TGF* transforming growth factor

Nevertheless, the magnitude of change in MPC surface marker expression induced by the PPS priming step for these three donors was found to be quite variable, suggesting that individual genetic variations may represent a dominant role. However, apart from STRO-1, the markers CD73, CD90, CD105, CD44, and CD146 were all observed to decline following PPS priming of the cells.

Human MSC monolayer cultures incubated with transforming growth factor (TGF)-beta for 7 days have been reported to undergo a similar downregulation of the surface antigens CD44, CD90, and CD105, a finding that was interpreted to signal an early phase of their de-differentiation to the chondrogenic phenotype [34]. We also observed a strong decline in CD146 antigen presentation on PPS priming, particularly for MPCs isolated from donor RAH3. The transmembrane protein CD146 is receptor highly expressed by endothelial cells [35] and on the surface of perivascular cells, which have recently been proposed as the source of MSCs within the perivascular niche of bone marrow [36]. Moreover, a recent study has provided compelling evidence that CD146 is a high-affinity netrin-1 receptor on endothelial cells [37]. Netrin-1 is a neuronal guidance molecule that promotes angiogenesis and vascular development of the endothelium following interaction with CD146 [36, 37]. In addition, expression of CD146 is associated with populations of human MPCs that promote the establishment of bone marrow elements, and enhance osteogenic differentiation and bone deposition when these cells are implanted subcutaneously into immune-deficient mice [38]. The present observation that CD146 expression by MPCs was markedly downregulated by PPS priming would therefore be consistent with our previous observations of reduced osteogenesis of MPCs when cultured or co-formulated with this agent in vitro [21] and in vivo [22–24].

Although many of the functions of the proteins encoded by the genes identified by RNA sequencing analysis could not be obviously assigned, the changes in the genes encoding the aggrecan core protein, IGF2, alpha chain type V collagen, FosB transgene, COMP, the proteinase ADAMTS4, and type II collagen alpha chains

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provided are consistent with increased chondrogenic differentiation of MPCs. For example, aggrecan core protein is necessary for the biosynthesis of PGs [39] and its upregulation is consistent with the known elevation of their biosynthesis by MPCs after PPS priming. The down regulation of the ADAMTSL4 gene could also be considered as beneficial for the deposition of a cartilaginous matrix as the protein it encodes is responsible for the degradation of PGs [39]. In addition, the upregulation of type V collagen could be significant as this protein is a contributor to the assembly of collagen fibres during cell growth and matrix assembly [40]. On the other hand, the downregulation of the COMP genes was unexpected since this protein is an abundant component of the cartilage extracellular matrix. However, studies with human MSCs have shown that enhancement of COMP gene expression did not increase the transcript levels of the chondrogenic markers Sox9 or aggrecan, suggesting that the role of COMP in matrix formation occurs at the post-transcriptional level [41]. Notably, the IGF2 gene was found to be strongly upregulated. As the proteins encoded by this gene play significant roles in the growth, differentiation, and survival of connective tissue cells, including articular cartilage [42], its elevation is consistent with the present study and our previous report on MPC chondrogenesis mediated by PPS [21]. The RNASeq-FastQ sequencing data also indicated that the FosB transgene was strongly upregulated by the priming process. Numerous studies have shown that the Fos genes are involved in the formation of heterodimeric complexes with members of the jun family of proto-oncogenes (c-jun, junB, jun D) to form the AP-I promotor complex required for gene transcription [43]. Following binding to consensus sequences in the regulatory regions of DNA, the Fos-Jun/AP1 complex mediates transcription pathways responsible for critical

cell functions, including differentiation and turnover of the extracellular matrix [44].

A related sulphated glycosaminoglycan, heparin, is known to bind and interact with a variety of cells where it also localizes in the nucleus and modifies gene expression [31, 32, 45–48]. Moreover, heparin has been used at low concentrations (<200 ng/ml) as a supplement for the culture expansion of embryonic stem cells [49, 50] and MSCs [51]. However, in a recent study which used human bone marrow-derived MSCs [52], it was demonstrated that when serial cultures of these cells were supplemented with heparin at a concentration equivalent to that used in the present study (500 µg/ml), cell growth was strongly retarded and MSC morphology and genetic expression modified to a senescent phenotype. These conflicting findings may be explained by the structural differences between these two polymers.

Like heparin, PPS is a poly-anion, but is not a glycosaminoglycan since it has a backbone structure consisting of repeating beta-D-xylanopyranose units to which a methyl glucopyranosyluronic acid ring is attached laterally every 9–10 xylanopyranoses units (Fig. 7). The xylanopyranose backbone required for the synthesis of PPS is extracted from Beech wood (*Fagus sylvatica*) hemi-cellulose, is first sulphate-esterified, and then fractionated to obtain the required molecular size. This semi-synthetic process affords a water-soluble poly-dispersed pharmaceutical preparation with a weight average molecular weight (MW) of 5700 Da and a high negative charge conferred by the large number of sulphate ester groups localised along its xylanopyranose backbone [53].

In contrast, native heparin is a structurally heterogeneous biopolymer that consists essentially of variably spaced repeating units of either 2-O-sulphated iduronic acid and 6-O-sulphated and N-sulphated glucosamine sugar rings linked glycosidically [54]. Commercially
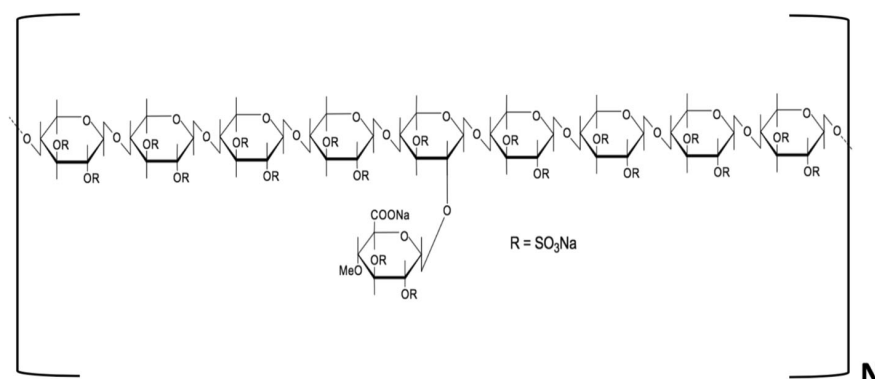

Fig. 7 Structural formula of the repeating unit of the poly-dispersed PPS. On average, a single sulphated 4-O-methyl-glucopyranosyluronic acid ring is attached laterally via an oxygen linkage to the 2 position of every sulphate-esterified 9–10th xylanopyranose unit of the polymer. From the molecular weight distribution of 1800–17,000 Da determined by size exclusion chromatography [44], N can be estimated as 0.5–6.0

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available heparin is more poly-dispersed than PPS with an averaged molecular weight ranging between 3000 to 30,000 Da [53] but is the most highly sulphated naturally occurring glycosaminoglycan with 2.7 sulphate groups/ disaccharide unit [54]. However, its charge density is less than that of PPS which on average contains 3–4 sulphate groups/disaccharide unit (Fig. 7).

Notwithstanding these significant molecular, charge, and conformational differences, PPS, because of its poly-anionic structure, does exhibit some heparin-like pharmacological activities. Although it is a weaker anti-coagulant than heparin, PPS is a strong fibrinolytic and lipolytic agent [52, 54]. These pharmacological activities resulted in its original clinical applications in the 1950s for the treatment of thrombotic and arteriosclerotic vascular disease [55]. However, over the intervening years, PPS has been shown to be effective for the management of more diverse medical indications, including interstitial cystitis [56], soft tissue inflammation [57], osteoarthritis [58–60], and Ross River Virus-related arthropathies [61].

In our earlier in-vitro studies, MPCs were cultured with PPS at various concentrations including 5.0 μg/ml, but for up to 10 days [21]. With the longer incubation periods, gene expression of Sox-9 and Aggrecan by MPCs was not significantly elevated relative to MPCs alone until day 7. In addition, expression of type II collagen was not significantly increased until day 10, when type X collagen, RUNX2, and Noggin gene expression was also suppressed [21]. These earlier RNA studies suggest that the present protocol of 24-h priming of MPC with PPS followed by maintaining cultures for up to 48 h prior to determination of gene expression may have been too short to establish the lifetime of genetic modifications. We therefore acknowledge that the maintenance of our PPS-primed MPC cultures for only 48 h represents a limitation of the present study. However, using an ovine model of disc degeneration induced by lumbar microdiscectomy we have demonstrated that PPS-primed MPCs when embedded in biodegradable collagen sponges implanted into the degenerate discs promoted the deposition of higher levels of proteoglycans and tissue repair after 6 months, compared with the injured disc injected with non-primed MPCs [62]. We consider that this in-vivo study supports our proposition that PPS-primed MPCs retained their modifying effects on gene and protein expression beyond the 48-h experimental period used in the present study.

Conclusions

These studies have shown that pre-incubation of MPCs with 5.0 μg/ml PPS for only 24 or 48 h was sufficient to invoke significant changes in their gene signature and protein expression consistent with enhanced proliferation and differentiation to the chondrogenic phenotype. The PPS priming step was undertaken at the penultimate phase of MPC culture expansion, a procedure that eliminated the necessity of combining the required quantities of the two agents at the time of clinical application and thereby eliminating the possibility that 'free' PPS was co-administered with the progenitor stem cells. Furthermore, from the results of the present study, together with the positive outcome of our animal model study [62], we conclude that pre-culturing of MSCs with agents such as PPS could provide an alternative method for reprogramming these cells to promote their differentiation towards a targeted phenotype that may be required for a specific medical indication, rather than their co-administration with agents that may independently be associated with undesirable side effects.

Additional file

Additional file 1: (A–E) Surface antigen expression of MPCs derived from three donors (RAH1, RAH2, and RAH) when cultured for 24 and 48 h with and without priming with 5.0 μg/ml PPS. Delta change represents the percentage change in antigen levels mediated by the PPS priming step. (DOCX 18 kb)

Ethics approval and consent to participate
Ethics approval and consent to participate was obtained from the Human Research Ethics Committee of the Adelaide Hospital.

Consent for publication
Not applicable

Competing interests
PG is a Director of Proteobioactives Pty. Ltd. but does not hold shares in the company. JW and SS were employees of Proteobioactives Pty. Ltd. but do not hold shares in the Company. The remaining authors declare that they have no competing interests.

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Appendix 2. Study Report: Covalently Bound PEG-Hyaluronic Acid-Pentosan Polysulfate Hydrogel ± Mesenchymal Progenitor Cells for Implantation Post Lumbar Discectomy

This appendix contains the unpublished manuscript of a collaborative experimental study entitled, **"Study report: Covalently bound PEG-hyaluronic acid-pentosan polysulfate hydrogel ± mesenchymal progenitor cells for implantation post lumbar discectomy"**. The manuscript describes the investigation of PEG-hyaluronic acid-pentosan polysulfate hydrogels, with/without mesenchymal progenitor cells, for the promotion of intervertebral disc regeneration following lumbar discectomy.

The candidate, Chris Daly, contributed to the concept, design, conduct and interpretation of experiments, writing and revising the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xxi.

Study Report: Covalently Bound PEG-Hyaluronic Acid-Pentosan Polysulfate Hydrogel ± Mesenchymal Progenitor Cells for Implantation Post Lumbar Discectomy

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Background

Microdiscectomy is one of the most commonly performed spinal surgical procedures. It is performed to treat pain or neurological deficit resulting from intervertebral disc degeneration and neural compression. Unfortunately, neither microdiscectomy nor any other clinically available therapy addresses the underlying pathology of disc degeneration. Consequently, up to one third of patients progress to experience chronic back pain(1) that may be disabling in up to 10% of those who have undergone microdiscectomy(2). Recurrent disc herniation occurs in up to 15% of patients and reoperation may be required in up to 25% of this cohort(3). Twelve percent of patients subjected to microdiscectomy will undergo reoperation for the same procedure within four years (4). Approximately 40% of these patients will subsequently progress to fusion surgery within four years (4).

Potential methods for treating the underlying process of disc degeneration are under investigation. One such approach, previously reported by our group(5), is the implantation of mesenchymal progenitor cells (MPC) combined with pentosan polysulfate (PPS) in a simple gelatin/fibrin scaffold. This approach

led to significant disc regeneration as evaluated by MRI, macroscopic and histological appearance and proteoglycan composition six months following microdiscectomy and matrix/MPC implantation. However, the gelatin/fibrin scaffold does not mimic the mechanical properties of the disc and has no special adhesive properties to ensure it integrates and remains within the intervertebral disc space providing the potential to mediate regeneration. It served primarily as a vehicle for administration of the MPCs and PPS into the excised disc space.

Hydrogels are highly hydrated polymeric materials that can be engineered to demonstrate mechanical properties similar to the components of the intervertebral disc. Through modulation of crosslinking kinetics, hydrogels can be injected into the defect of the intervertebral disc prior to gelation creating a mold the exact size of the discectomy defect that subsequently hardens. The cross-linked hydrogel should maintain disc height and eliminate the risk of the implanted matrix and MPCs being displaced from the disc space. Hydrogels may also be loaded with biologically active molecules and previous studies have already demonstrated successful incorporation of PPS into a hydrogel scaffold populated with MPCs leading to increased Collagen Type II production(6,7). As such the combination of hydrogels with PPS and MPCs suggests promise for inducing regeneration in the post-microdiscectomy intervertebral disc.

Objective

The objective of this study is to evaluate the potential of a novel polyethylene glycol (PEG) - hyaluronic acid (HA) - covalently bound pentosan polysulfate (PPS) (PEG-HA-PPS) hydrogel matrix alone and in combination with allogeneic bone marrow derived STRO-3$^+$ immunoselected mesenchymal progenitor cells (MPCs) to facilitate lumbar intervertebral disc regeneration in a post-microdiscectomy sheep model.

Methods

Surgical Procedure

With ethics approval from the Monash Medical Centre Animal Ethics Committee, 18 adult (two to four years of age) Border-Leicester Merino cross-bred ewes underwent preoperative 3 Tesla MRI (Siemens Skyra Widebore 3T MRI, Siemens, Erlangen, Germany) under general anesthetic. Sheep ambulated freely prior to the trial and were raised in open pastures. All sheep were fasted for 24 hours prior to surgery and anesthetized using intravenous thiopentone (10-15mg/kg) (Bayer Australia Ltd., Pymble, NSW, Australia) followed by intubation and isoflurane inhalation (Pharmachem, Eagle Farm, QLD, Australia) (2-3% in oxygen). Sheep were placed in the right lateral position. Local anesthetic (bupivacaine 0.5%)(AstraZeneca Australia, Macquarie Park, NSW, Australia) was administered subcutaneously and the L2-3 and L3-4 lumbar intervertebral discs exposed via left lateral

retroperitoneal approach, as previously described(8,9). Intraoperative lateral radiographs (Radlink, Atomscope HF200A, Redondo Beach, CA, USA) were performed to confirm the correct levels. A standardized microdiscectomy procedure, utilizing a drill bit, was performed at two consecutive lumbar disc levels: L2/3 and L3/4. Standardization of the extent of the microdiscectomy was achieved by using a 3.5mm Brad point drill bit (Carbatec, Melbourne, Victoria, Australia) with a drill bit stop applied at 12mm drill bit length (Drill Warehouse, Amazon, Seattle, USA) as described previously(9,10).

Following standardized discectomy sheep were randomised into the following three experimental groups of six sheep each:

A. Microdiscectomy injury only

B. Microdiscectomy injury with injection of activated PEG-HA-PPS hydrogel (no MPCs)

C. Microdiscectomy injury with injection of activated PEG-HA-PPS hydrogel with MPCs

The adjacent L1/2 and L4/5 levels served as normal controls. This is illustrated below in **Figure 1**.
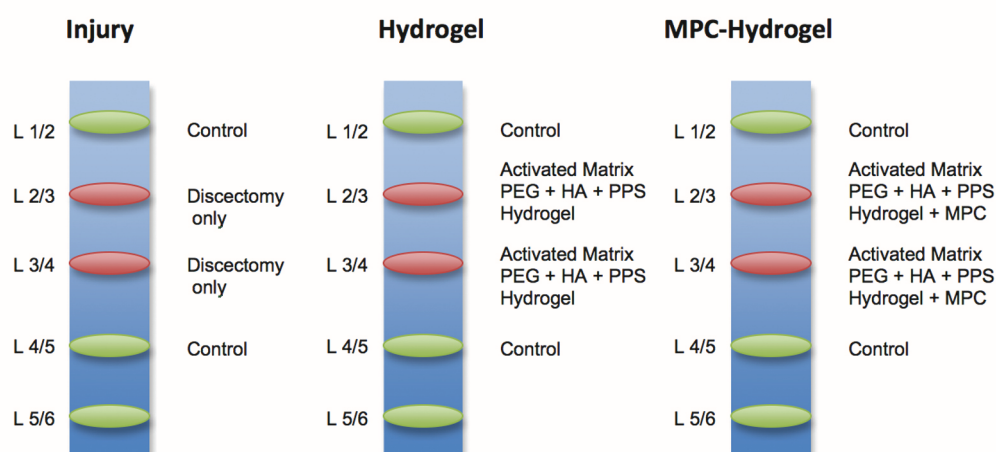

Figure 1. Experimental groups Sheep were randomly allocated to the above three groups. The L2/3 and L3/4 intervertebral disc served as the intervention discs with the adjacent L1/2 and L4/5 discs controls.

Injury animals (n=6) underwent drill bit injury only at the L2/3 and L3/4 levels. Hydrogel animals (n = 6) received activated cross-linked PEG-HA-PPS hydrogel. MPC-Hydrogel animals received activated PEG-HA-PPS hydrogel cross-linked with 0.5×10^7 MPCs contained with the hydrogel.

Hydrogel Preparation

The hydrogel used in this investigation was provided by the laboratory of Prof. Justin Cooper-White (Australian Institute of Bio and Nanotechnology (AIBN), University of Queensland) with the aid of Dr Taryn Naidoo (AIBN).

The preparation of the hydrogel has been described in detail previously(6,7). The hydrogel, per 1ml, consisted of the following:

Hyaluronic Acid-Tyramine (HA-TYR)(25mg/ml in Phosphate Buffered Saline(PBS))	600µl
Polyethylene Glycol 3-4 hydrogephenyloprionic acid (PEG-HPA)	
(200mg/ml in PBS)	82.5µl
Hyaluronic Acid-Pentosan Polysulfate (HA-PPS) (1mg/7.8ml)	125µl
Phosphate Buffered Saline	80µl
Horseradish Peroxidase (10µl/ml)	12.5µl
Hydrogen Peroxide (100mmol) (added to commence gelation)	100 µl

The above components, with the exception of hydrogen peroxide, were combined in proportion to produce ~900µl of hydrogel stock solution. Hydrogen peroxide was added later at the appropriate ratio at the time at which gelation was desired to commence. This produced gelation at approximately 5-6 minutes with maximum gelation at approximately 11 minutes.

MPC-Hydrogel Preparation

The MPC-hydrogel injection was prepared as follows:

1. MPCs were contained within ampules of 2.5×10^7 MPCs at a concentration of 5×10^7/ml with 0.5ml per ampule.
2. Vials were thawed in 37C water bath and premixed to ensure cells were evenly distributed.
3. A 100 µl aliquout was removed from the ampule for the cell count.
4. The 100 µl aliquot was diluted 1/10 with the supplied SF Alpha MEM.
5. A further ½ dilution in trypan blue was be performed.
6. Cell count and viability check was performed on the 1/20 diluted solution (SF Alpha MEM and Trypan Blue).

7. The remaining 400μl solution was centrifuged, supernatant removed and 400μl hydrogel solution added.

8. The MPCs and hydrogel solution was mixed under sterile conditions in a laminar flow cabinet.

9. After the intervertebral disc injuries were made and prepared for injection the hydrogen peroxide was added to the mixed hydrogel-MPCs to initiate gelation.

10. The mixed materials were drawn up into two sterile empty 1 ml syringes with 18G drawing up needles~240μl per tube.

11. The intervertebral discs were injected with ~120μl of solution prior to gelation (requested gelation time approximately 10 mins).

Following drill bit discectomy, +/- hydrogel or MPC-hydrogel administration, the wound was closed via a routine layered procedure using absorbable sutures (Vicryl, Ethicon, NJ, USA). Animals received a fentanyl patch (Duragesic 75 μg/hr, Jannsen LLC., North Ryde, NSW, Australia) and intravenous paracetamol (Pfizer Ltd., West Ryde, NSW, Australia) for postoperative analgesia. Animals were recovered and returned to the pen with other sheep and allowed free ambulation. One week post-surgery sheep returned to open pasture.

Necropsy

Animals were euthanized by intravenous injection of 150 mg/kg of pentobarbital (Sigma-Aldrich, Castle Hill, NSW, Australia) six months post-surgery. The lumbar spines were removed *en bloc*, a segment was isolated from the mid-sacrum to the thoracolumbar junction, and transferred to Monash Biomedical Imaging for MRI analysis. The lumbar spines were then transected in the horizontal plane through their vertebral bodies, to provide spinal segments consisting of a complete lumbar disc with half of the adjacent vertebral bodies attached. Subsequent gross morphological, biochemical and histological analysis of discs were undertaken as described below. Spinal segments containing discs destined for histological analysis were transferred to phosphate buffered formalin.

Radiological Analysis

Using standard digital processing software (Osiris MD v8.0.2, Pixmeo, Geneva, Switzerland) disc height index (DHI) measurements were calculated and recorded by an observer blinded to the treatment regimen. Disc height index analysis was performed using the pre-operative and 3T MRI images obtained at necropsy, by an observer blinded to the intervention protocol. The use of 3T MRI eliminated the potential for parallax error while also producing consistent image quality for all discs.

Axial 9.4T (Agilent 9.4T MRI Small Animal Scanner Agilent/Varian, Santa Clara, CA, USA) T1 and T2 MRI sequences of the lumbar intervertebral discs were taken for each animal. Sagittal T2 weighted 3T

(Siemens Skyra Widebore 3T MRI, Siemens, Erlangen, Germany) sequences of the entire lumbar spine explant were obtained for each animal. Pfirrmann MRI disc degeneration scores for all lumbar discs were determined using sagittal 3T T2-weighted sequences and 9.4T T2 sagittal reconstructions (Osiris MD v 8.0.2) by four blinded observers (a neuroradiologist, neurosurgeon and two neurosurgery residents blinded to the treatment regimen).

Gross Morphological Analysis

Lumbar spinal disc segments allocated for biochemical and gross morphological analysis were sectioned in the horizontal (axial) plane to provide two complementary halves of the disc as shown diagrammatically in **Figure 2**. High resolution digital photographs were taken of the exposed complementary surfaces. Each region shown in **Figure 2** was scored by a blinded observer following the criteria in **Table 1** described by Daly et al.(11) and adapted from the method of Oehme et al.(12).
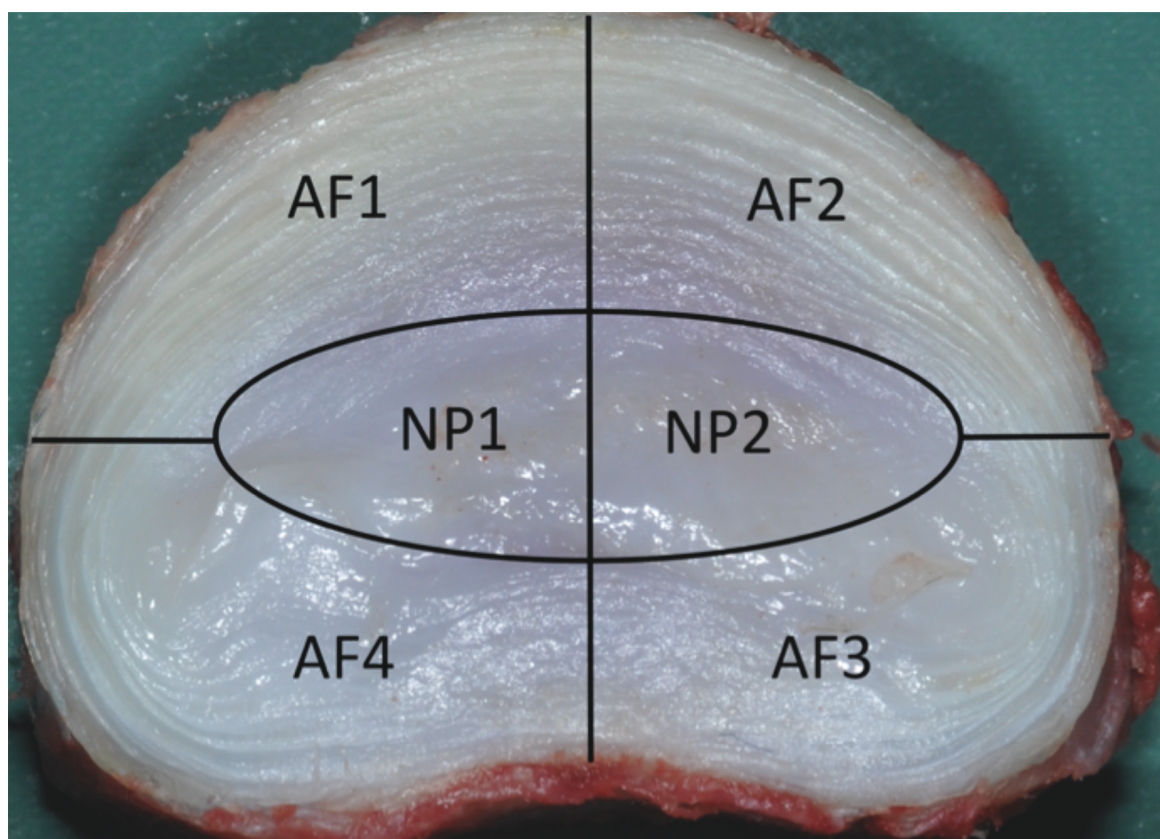

Figure 2. Diagram demonstrating intervertebral disc segments used for gross morphological and biochemical analysis. AF1 is the site of intervertebral disc drill bit injury. NP1 is the region of NP on the injured half of the intervertebral disc. NP2 is the complementary half of NP1. AF, annulus fibrosus; NP, nucleus pulposus. Image from Daly et al.(11)

Table 1. Gross morphology criteria used to score segmental regions (AF and NP) shown in Figure 2 for each disc*

AF Morphological grades applied to each AF quadrant	NP Morphological grades applied to each half of NP.
Grade 0: Normal Disc Normal disc, no annular disruption, discoloration or hemorrhage.	**Grade 0: Normal NP** No discoloration or hemorrhage
Grade 1: Minor Disruption Annular disruption with minor discoloration and/or hemorrhage	**Grade 1: Minor Disruption** Minor disruption, discoloration and/or hemorrhage. <10% NP region. Minor fissuring and nuclear dehydration may be evident.
Grade 2: Moderate Disruption Annular disruption with medium discoloration and/or hemorrhage.	**Grade 2: Moderate Disruption** Medium disruption, discoloration and/or hemorrhage. 10-50% of NP region. Moderate fissuring and nuclear dehydration may be evident.
Grade 3: Major Disruption Annular disruption with significant discoloration and/or hemorrhage.	**Grade 3: Major Disruption** Significant disruption, discoloration and/or hemorrhage. 50-75% NP region. Major fissuring and nuclear dehydration may be evident.
Grade 4: Complete Disruption: Annular disruption with extensive discoloration and/or hemorrhage.	Grade 4: Complete Disruption: Extensive disruption, discoloration and/or hemorrhage. >75% NP region. Extensive fissuring and dehydration may be evident.

*The sum of all regional scores (AF1, AF2, AF3, AF4, NP1 and NP2) yielded a total disc degeneration score between 0 (normal) and 24 (severely degenerated) for each disc. (AF=Annnulus Fibrosis, NP=nucleus pulposus). Table is described in Daly et al.(11) and adapted from the method described by Oehme et al.(12).

Biochemical Analysis

Following collection of disc digital images for morphological analysis all tissue regions were subjected to biochemical analysis. The individual annulus fibrosus (AF) and nucleus pulposus (NP) from each region were separated from each other and their vertebral attachments by careful dissection using the boundaries shown in Figure 1. Tissues from each region were finely diced, frozen in liquid N2 and powdered. The powdered tissues were transferred to pre-weighed Eppendorf vials and weighed, lyophilised and reweighed to constant weight to determine their anhydrous weights. Aliquots of the dehydrated tissues, in triplicate, were solubilized using a papain digestion buffer (50 mM sodium acetate [pH = 6.0]) containing 2mg/ml papain (Sigma-Aldrich Chemicals, Sydney, NSW, Australia) by incubation at 60°C for 16 hours (13). The digested tissues were centrifuged for 15 minutes at 3000g and supernatants diluted to standard volumes. Aliquots of the stock solution were analysed for sulphated glycosaminoglycan (S-GAG) (an index of proteoglycan content) levels using the dimethylmethylene blue (DMMB) assay(14), hydroxyproline assay (to derive collagen content)(15) and Hoechst dye 33258 assay for DNA content(16). The results of biochemical analyses were normalized and were expressed as percentage of tissue dry weight for S-GAG, collagen and DNA.

Histological Analysis

The individual disc segments, consisting of the intervertebral disc with attached hemisected vertebral bodies were in 10% neutral buffered formalin for eight days then stored in 70% ethanol. The volume of vertebral bone was reduced to the growth plate using a fine diamond saw. Prior to paraffin based tissue embedding, decalcification of the remaining vertebral bone was undertaken with multiple changes of 10% formic acid. Segments were bisected in the sagittal plane and then axial paraffin sections were cut using a standard rotary microtome and stained using Haematoxylin & Eosin and Safranin-O/Fast-Green. Axial sections were taken to allow for visualization of the entire drill bit injury tract.

Statistical Analysis

All data analysis and storage was performed using Microsoft Excel for Mac (Version 15.33, Microsoft, Redmond, WA, USA) and Prism 7.0c for Mac (GraphPad Software Inc., La Jolla, CA, USA). Parametric data were analyzed using one-way ANOVA, and the Tukey's multiple comparison test was performed when significant differences in means were observed. Nonparametric data were analyzed using Kruskal-Wallis test of median values followed by Dunn's multiple comparison test. Groups were compared using the two-tailed Student t-test followed by Mann-Whitney U-tests. A p value < 0.05 was considered statistically significant.

Results

Disc Height Index

Drill injured, hydrogel and MPC-hydrogel discs all demonstrated significantly increased disc height index loss compared to control discs ($p<0.001$)**(Figure 3A)**. Baseline pre-operative 3T MRI revealed no evidence of pre-existing intervertebral disc degeneration at the intervention or control levels. 3T MRI Pfirrman grades were significantly increased in the injured, hydrogel and MPC hydrogel discs relative to the control discs ($p<0.001$) with no significant differences among the intervention and injured discs **(Figure 3B & 4)**.
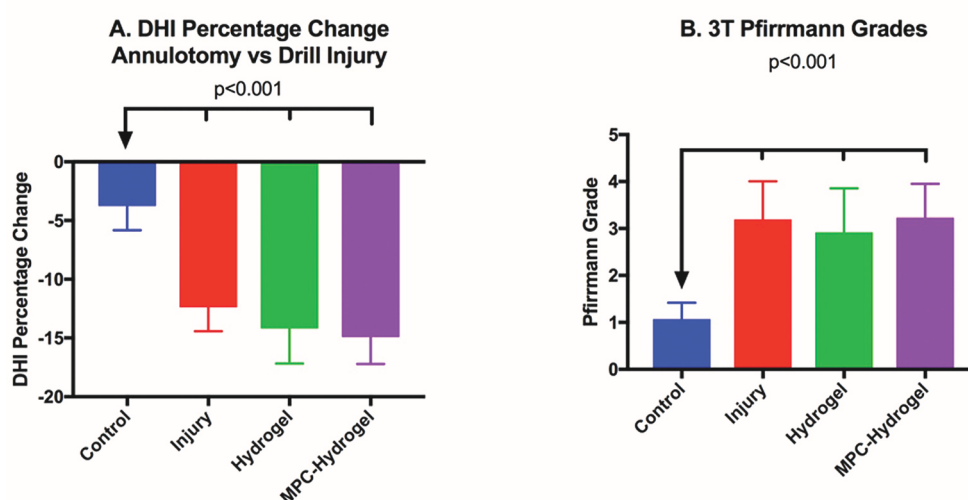
Figure 3. 3T MRI A. Disc height index percentage change all injured and treated discs demonstrated increased DHI loss relative to controls with no significant difference among groups. **B. 3T Pfirrmann grades** were also significantly increased in the in the injured and treated disc relative to controls.
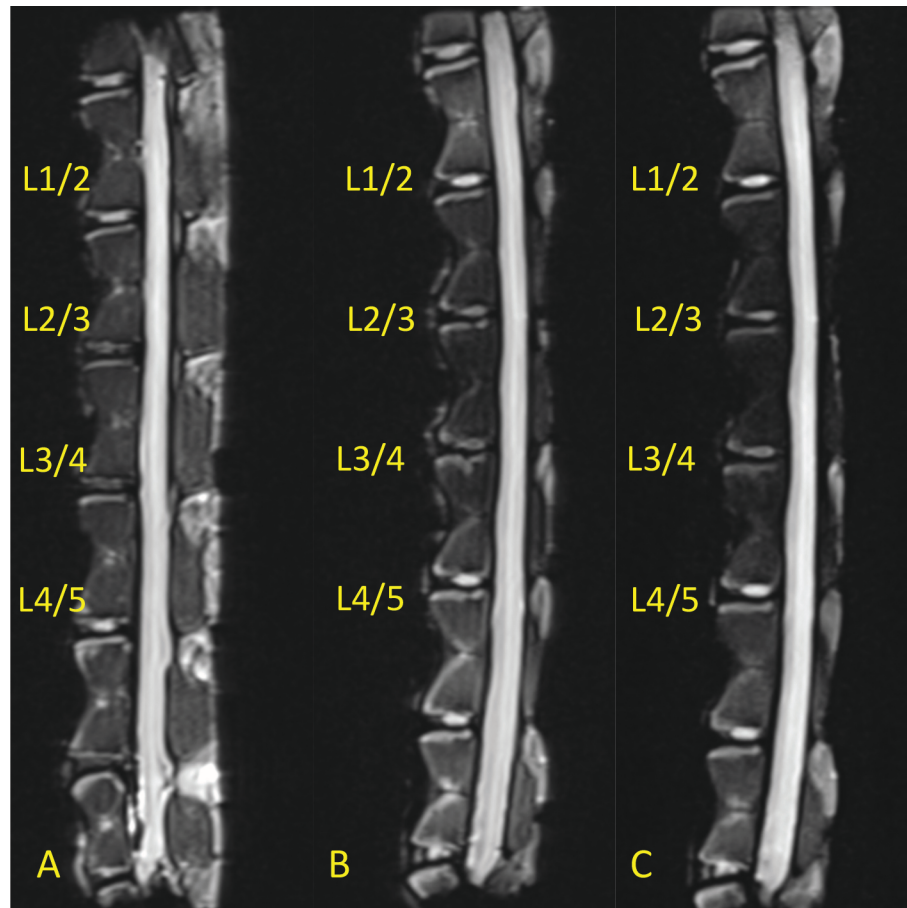
Figure 4. Post operative 3T MRI T2 sagittal images. A. Injury discs demonstrating increased Pfirrmann grade and disc height loss as L2/3 and L3/4 injured discs relative to L1/2 and L4/5 control discs. **B. Hydrogel discs** and **C. MPC-Hydrogel discs** demonstrate the same pattern.

9.4T MRI axial imaging allowed better appreciation of the focal nature of the drill bit injury **(Figure 5. B, C & D)**. Pfirrmann grades on 9.4T MRI were significantly elevated for the injury, hydrogel and MPC-hydrogel discs relative to the control discs (p<0.001) with no significant differences among the injured and treated discs **(Figure 5. E)**.
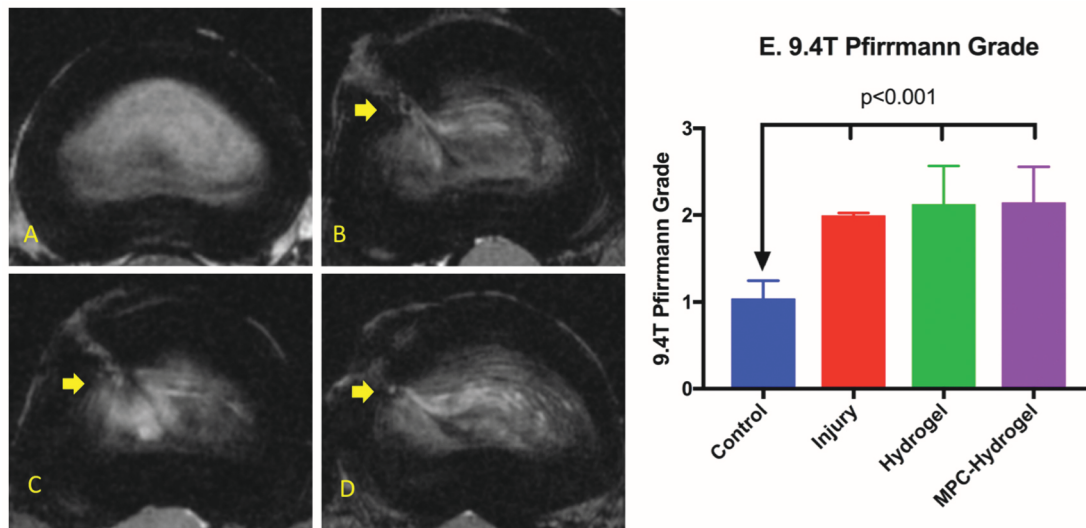
Figure 5. 9.4T MRI T2 axial images of A. Control demonstrating hyperintense intact NP in contrast to **B. Injury, C. Hydrogel** and **D. MPC‑Hydrogel discs** demonstrating injury tract extending through AF to NP with reduced NP hyperintensity. This is reflected in **E. 9.4T Pfirrmann grades** in which the injured and treated discs demonstrated significantly increased 9.4T Pfirrmann grades relative to the control discs.

Gross Morphology

Gross morphological analysis was completed on a regional basis with the data presented in **Figure 6E** showing the aggregate morphological scores. Representative gross morphological images are displayed in **Figure 6A, B, C & D** and their respective scores, determined using the criteria displayed in Table 1 are shown In **Figure 6E.** Control discs demonstrate an intact AF and NP with a homogenous white appearance. Injured **(Figure 6B)** and treated discs **(Figure 6C & D)** demonstrate the drill injury tract extending through the AF into the NP, disruption of the AF adjacent to the tract with discolouration of the NP. Total disc gross morphological scores were significantly elevated in the injury, hydrogel and MPC‑hydrogel discs relative to controls ($p<0.001$) with no significant difference amongst these groups **(Figure 6E)**.
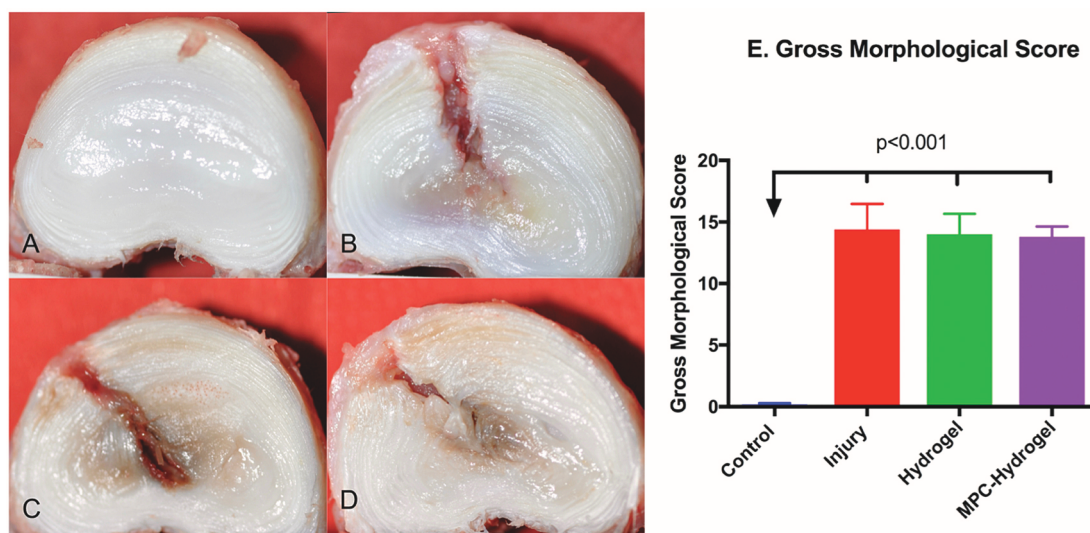
Figure 6. Representative examples of disc gross morphology A. Control disc demonstrating intact AF and NP. **B. Injury disc, C. Hydrogel disc** and **D. MPC-Hydrogel discs** demonstrating injury tract extending through AF into NP with NP discolouration. **E. Gross morphological scores** demonstrate significant increased scores in the injured and treated discs relative to the control discs with no significant difference among these groups.

Proteoglycan content as determined by Sulfated-Glycosaminoglycan (S-GAG) analysis

Sulfated-glycosaminoglycan content was significantly reduced in the NP-1, NP-2 and NP-Total **(Figure 7 A, B & C)** compared to controls (p<0.005) with no significant differences amongst these groups. AF-1 S-GAG content was also significantly reduced in the hydrogel discs relative to the control discs. No other significant differences were observed on S-GAG analysis.
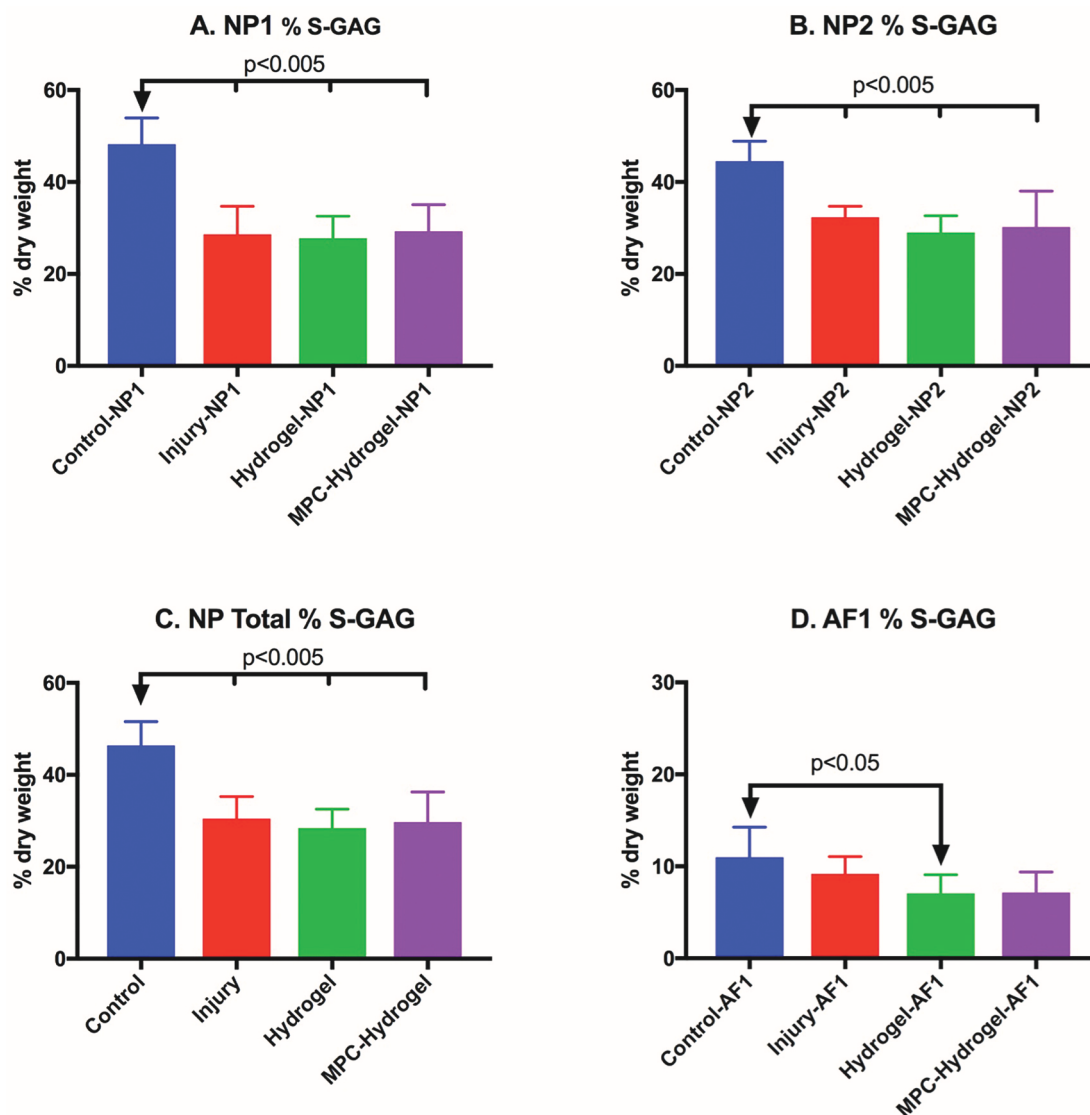
Figure 7. Sulfated-GAG content (%dry weight). A. NP1 % S-GAG was significantly lower than control discs in injury, Hydrogel and MPC-Hydrogel discs. This relationship was observed for **B. NP2 % S-GAG** and **C. Total % S-GAG. D. Hydrogel AF-1** was significantly lower than control AF-1.

Collagen Content

The NP collagen content of the MPC-hydrogel discs was significant increased relative to the control discs ($p < 0.05$) **(Figure 8)**. No other significant differences were observed on collagen analysis.
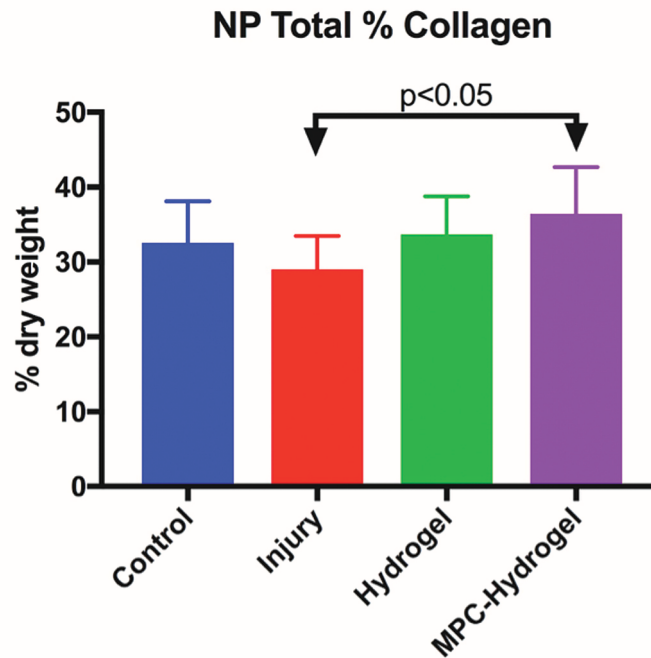
NP Total % Collagen

Figure 8. Collagen content (% dry weight). Collagen content was significantly increased in the MPC-Hydrogel discs relative to the injury discs.

DNA analysis did not reveal any significant differences among the groups.

Histology

Unfortunately, due to technical challenges in histological processing, sufficient adequate histological specimens were not obtained to allow quantitative analysis to be performed. Qualitative histological analysis revealed that injury, hydrogel and MPC-hydrogel discs all demonstrated the drill injury tract extending through the AF into the NP with reduced NP Safranin-O staining compared to control discs. No significant differences in the histological appearance among the injury, hydrogel and MPC-hydrogel discs could be discerned.
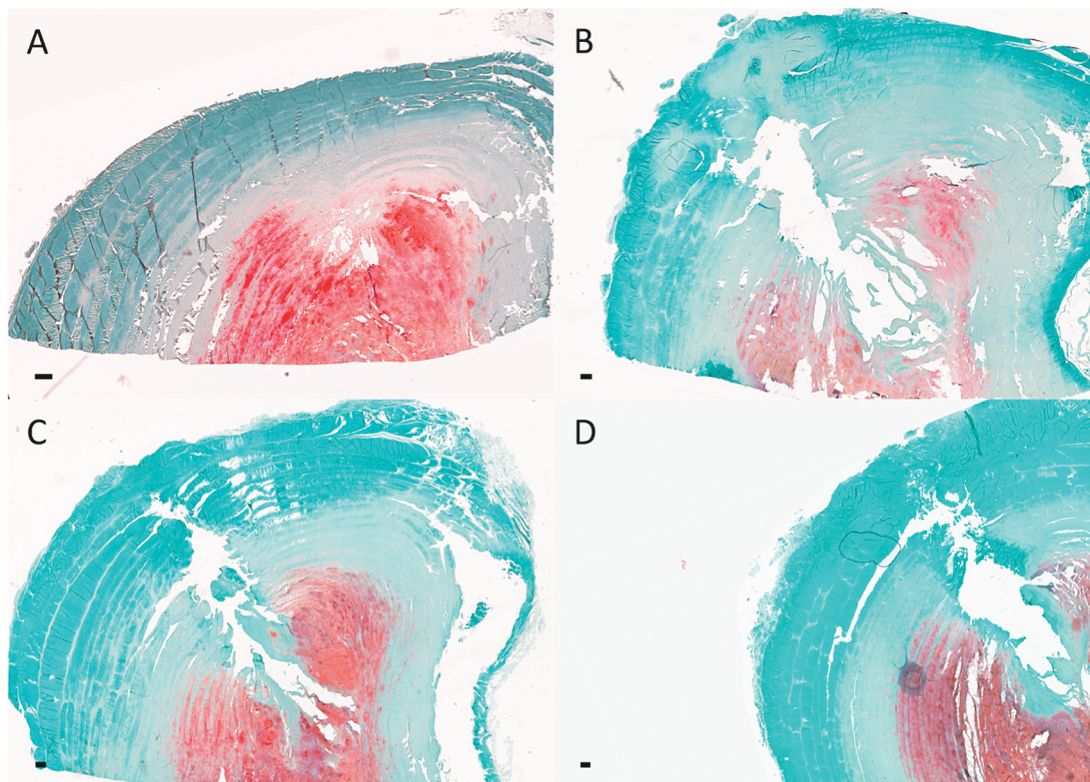
Figure 9. Sections stained with Safranin O and Fast Green. A. Control disc demonstrating intact annulus fibrosus and adjacent nucleus pulposus. **B. Injury disc** demonstrating drill injury tract extending through annulus fibrosus into nucleus pulposus with reduced NP Safranin-O staining. **C. Hydrogel disc** and **D. MPC-Hydrogel disc** demonstrated a similar pattern of NP and AF injury with reduced NP Safranin-O staining. (Scale bar = 500 μm).

Discussion

The results of the present study demonstrated that the drill bit injury induced intervertebral disc degeneration in an ovine model. However, neither the hydrogel or MPC-hydrogel treated discs displayed evidence of significant intervertebral disc regeneration as assessed by gross morphological, radiological, biochemical or histological analysis. Indeed, the only significant difference observed among the injured and treated groups was the significant increase of nucleus pulposus collagen observed in the MPC-hydrogel discs compared to the injured discs. Additionally, the hydrogel discs demonstrated a significant reduction in AF-1 sulphated glycosaminoglycan content relative to the control discs, however, they did not differ from injury or MPC-hydrogel discs.

The observation that NP collagen was increased in the MPC-hydrogel treated discs could be interpreted as evidence of enhanced healing in terms of a fibrotic response that could potentially reduce the risk of further intervertebral disc herniation. Intervertebral disc reherniation following lumbar discectomy is a significant cause of post-operative morbidity, with 12% of patients undergoing reoperation for intervertebral disc reherniation within four years of their index procedure(3). Various

attempts to repair the annulus fibrosus following microdiscectomy have been investigated including direct suture(17), insertion of amniotic membrane(18) or annular closure prostheses(19). One such prosthesis(19) has demonstrated promising in pilot clinical studies. The increase in collagen in the MPC-hydrogel discs observed in this study may potentially offer benefits in terms of reducing reherniation. However, given the lack of formal biomechanical assessment of the intervertebral discs or longer term radiological follow-up for evidence of reherniation, such therapeutic effects remain theoretical and warrant further investigation.

The significant difference between hydrogel disc AF-1 S-GAG content and control discs, and the absence of such a difference between the injury and MPC-hydrogel discs, is more difficult to explain. The presence of MPCs within the MPC-hydrogel could potentially explain the absence of a difference from controls, given prior demonstration of the ability of MPCs in a PEG-HA-PPS hydrogel to secrete proteoglycan(6). However, this does not explain the lack of any difference between the injury discs and control or the lack of any other evidence of regeneration in the MPC-hydrogel discs other than increased nuclear collagen. PPS, independent of MPCs, has been demonstrated to support chondrocyte and fibroblast anabolic activity while attenuating cartilage matrix catabolism(20) and as such is unlikely to be the cause of the reduction in AF-1 S-GAG. Similarly, hyaluronic acid has also been demonstrated to promote intervertebral disc collagen I synthesis and downregulate matrix catabolic processes(21). As such, the observation of decreased hydrogel AF-1 S-GAG content relative to controls remains difficult to explain.

MPCs have been demonstrated to promote intervertebral disc regeneration in a variety of ovine models of disc degeneration including partial thickness annulotomy(12), microdiscectomy(5) and chondroitinase-ABC models(22) at doses comparable to those used in this study (0.5×10^7). Preliminary large animal studies of the application of hydrogels in the treatment of intervertebral disc degeneration have demonstrated short term intradiscal hydrogel retention and delivery of contained therapeutic agents(23-25). As such the failure of the hydrogel and MPC-hydrogel treated discs to demonstrate evidence of significant regeneration six months following injury and administration raises the question of the cause of this disappointing outcome.

Given the use of hydrogen peroxide to promote hydrogel crosslinking compromise of MPC viability bears consideration as a contributor to the lack of intervertebral disc regeneration. MPC viability following hydrogen peroxide has previously been demonstrated *in vitro* at seven days in the PEG-HA-PP hydrogel used in this study(6). Furthermore, in the same study, proteoglycan and collagen production was confirmed histologically at 21 days, indicating the MPCs were displaying some chondrogenic activity. The method used to promote hydrogel crosslinking in our study was that described by Frith et al.(6) and as such MPC viability was expected, although was not confirmed as

part of our protocol. The MPCs used in the study of Frith et al.(6) were of human origin, whereas ovine MPCs were used in our study, and although this is unlikely to explain the difference in outcome it is a point of difference that bears mention.

The ovine drill bit injury model is novel relative to the more commonly used annulotomy models of intervertebral disc degeneration described above and as such could be hypothesized to be another confounding variable in this study. However, the drill bit injury model adequately induced lumbar intervertebral degeneration, as was evident on radiological, morphological, biochemical and histological analysis. Furthermore, the extent of degeneration induced is less advanced than other annulotomy models(5), as we have recently demonstrated. As such this model may more readily allow assessment of cellular therapies suited to treat early degeneration in which the interaction of administered therapies with native disc cells may play a significant role. The absence of any significant differences among the injured and treated groups, other than the increased NP MPC-hydrogel disc collagen detailed above, indicates that this was not the case in our study for reasons that are not clear.

Significant leakage of cellular therapies administered to the intervertebral disc has been demonstrated by other investigators and may be a significant contributor to the outcome observed in this report. Omlor et al.(26) demonstrated leakage of 90% of mesenchymal stem cells three days following implantation into partial nucleotomised porcine intervertebral discs. Although the presence of hydrogel at the injury site was visually confirmed at the end of the procedure some leakage was observed during administration. Furthermore, in the absence of cell labelling and interval re-imaging, there was no way to confirm persistence of the hydrogel in the intervertebral disc following surgery and animal recovery. Thus, leakage of the hydrogel or MPC-hydrogel following intervention remains a significant potential contributor to the results observed in this study.

Conclusions

In conclusion, this study did not demonstrate any significant regenerative effect following administration of a PEG-HA-PPS hydrogel, with or without MPCs, after drill bit injury induced intervertebral disc degeneration in an ovine model. Given the previous promising results observed in vitro for the PEG-HA-PPS-MPC hydrogel and other large animal studies investigating MPCs and hydrogels for intervertebral disc degeneration, the results observed in this study were disappointing. However, this disappointment must be tempered by acceptance of the potential contributions of the limitations described above to this initial large animal study.

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Appendix 3.   Back pain, the opioid crisis and novel alternatives

This appendix contains the manuscript entitled, **"Back pain, the opioid crisis and novel alternatives"**. The manuscript is a perspective paper exploring the potential of mesenchymal progenitor cells as a novel alternative therapy for back pain in the context of the opioid crisis enveloping North America and, to a lesser extent, Australasia. This manuscript has been submitted for consideration to the ANZ Journal of Surgery.

The candidate, Chris Daly, contributed to the concept, design, writing and revising the manuscript. Proportional contributions of co-authors are explained in the signed declaration on page xxii.

Full Title: Back pain, the opioid crisis and novel alternatives

Running head: Back pain, opioids and stem cells

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This paper is original and not based on any prior communications

This paper includes no figures or tables and, as a perspective paper, does not include an abstract

Word Count: 1143

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North America is in the grip of an opioid crisis. The sales of prescription opioids have quadrupled over the last two decades, along with the rates of misuse, overdose and death. Estimates of the financial burden attributable to opioid use range between $560 billion to $635 billion a year in the United States alone[1]. The opioid crisis has not left Australia unscathed. Opioid use in Australia has also quadrupled between 1990 and 2014 with the use of long-acting opioids increasing 17-fold between 1990 and 2000[2]. Opioid related harms and cost to the Australian community have also dramatically increased, and hospitalizations attributable to opioids have more than doubled between 1998 and 2009, overtaking heroin overdoses in 2001.

Low back pain (LBP) is the most common form of non-cancer pain for which opioids are prescribed, accounting for about 30% of its use, and opioid prescription rates for LBP are increasing[2]. LBP is the leading cause of disability worldwide and the third costliest medical condition in the United States. LBP will be experienced by 75-80% of people at some stage in life. Disc degeneration is strongly associated with LBP.

Definitive treatment of LBP remains an elusive goal. A significant proportion of patients fail conservative management and, despite guidelines to the contrary, many commence long term opioid therapy. Surgery offers no panacea for LBP. The use of lumbar fusion for the treatment of discogenic LBP remains contentious. Randomised controlled trials (RCTs) investigating these therapies have produced mixed results. Improvements in pain and disability following lumbar fusion for LBP have been demonstrated[3], however, a similar number of RCTs have demonstrated no significant difference between surgery and conservative management[4]. Notwithstanding the contention, lumbar fusion fails to treat the underlying pathology. Thus, existing therapies for low back pain are inadequate.

Given the failings of conventional treatment novel therapies for LBP are under investigation worldwide. Autologous disc chondrocytes were the first cellular therapy investigated for disc degeneration[5]. Disc chondrocytes were collected at the time of lumbar discectomy, expanded in cell culture and subsequently reimplanted percutaneously into the symptomatic disc 8-12 weeks following surgery. This therapy demonstrated promise, with many patients remaining symptom free and experiencing functional recovery. However, this approach has significant limitations. Metachronous invasive procedures are required for surgical cell harvest and subsequent reimplantation. Appropriate cell culture facilities may be prohibitively expensive and are not available in many institutions. Thus, widespread application of this approach is unlikely.

In light of these difficulties allogeneic mesenchymal progenitor cells (MPCs) bear particular mention. MPCs are the major colony forming population present within the bone marrow, retain extensive proliferative capacity, have greater plasticity than mature stromal cells and are immunoprivileged,

enabling them to be used in an allogeneic fashion. These cells have demonstrated significant potential in the promotion of intervertebral disc regeneration in extensive preclinical and clinical studies[6]. In large animal studies, MPCs have been demonstrated to promote intervertebral disc regeneration as assessed by radiological, gross morphological, biochemical and histological analysis[7,8]. Phase II clinical trials have demonstrated the ability of these cells, when administered percutaneously, to significantly reduce low back pain, disability, opioid consumption and rates of reintervention[9].

We recently demonstrated in a large animal model that the efficacy of MPCs in promoting disc regeneration is further increased by cell "priming" prior to administration. MPCs were cultured with pentosan polysulfate (PPS), a clinically approved polysulfated polysaccharide, for 24 hrs prior to administration into the intervertebral disc. PPS promotes the proliferation and chondrogenic differentiation of MPCs *in vitro* and *in vivo*[11]. Discs treated with PPS primed MPCs demonstrated reduced vascular and cellular infiltration with increased proteoglycan content relative to MPC treated discs. Given the integral role of neurovascular invasion and inflammation in the pathogenesis of discogenic back pain, these histological and biochemical findings suggest potential additional benefits attributable to PPS priming in the treatment of discogenic pain. This promising novel therapy will be investigated in a pilot clinical study in the near future.

MPCs are hypothesized to have their effect on intervertebral disc regeneration and back pain via one or more of the following major mechanisms of action: local survival of MPCs with differentiation towards chondrogenic cells, release of trophic factors influencing the local milieu of resident cells and via an anti-inflammatory effect. These cells have additional effects including the down regulation of primary nociceptive afferent input[12]. A Phase 3 Study investigating MPCs for the treatment of discogenic back pain is almost complete and is the final hurdle before these cells become a clinically available treatment for LBP.

Conclusion

The rising prevalence of low back pain combined with the inadequacies of existing therapies and the morbidity of inappropriate opioid usage, demand the development of alternative treatments for this condition. MPCs have demonstrated significant promise in preclinical and clinical studies and their translation to clinical application presents an opportunity to reduce the burden of low back pain and associated opioid misuse.

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