Clinically quantifiable measures from an *in* vitro study of nucleus augmentation of the intervertebral disc

Dixon A.R.[1], Warren J.P. [1], Culbert M.P. [1], Khan A.[2], Mengoni M. [1], Wilcox R.K. [1*]

[1]University of Leeds, Institute of Medical and Biological Engineering, Leeds, LS2 9JT, United Kingdom

[2]Consultant spine surgeon, Leeds Centre for Neurosciences, Leeds Teaching Hospitals NHS Trust

*Corresponding Author: r.k.wilcox@leeds.ac.uk

Abstract

Purpose: Nucleus augmentation has been proposed as an early-stage intervention for intervertebral disc degeneration and involves the injection of a biomaterial into the nucleus to restore disc height and functionality. The aim of this work was to identify clinically relevant quantitative measures that indicate the mechanical performance of the disc following nucleus augmentation.

Method: Bovine tail bone-disc-bone units (n=22) were mechanically tested under cyclic loading sequentially in native, artificially degenerated and treated states. Treatment involved injection of a peptide:glycosaminoglycan mixture into the degenerated disc to a predetermined load using a syringe driver with an integrated force sensor. The mechanical restoration of the treatment was determined by comparing the biomechanical behaviour of the native state to the treated state of each disc. The mechanical restoration was then compared against clinically quantitative parameters.

Results: Significant biomechanical differences were observed from the degenerate state to the native and treated states. The force delivered during injection was found to ramp to a steady state, followed by a final rapid increase, however all measures associated with injection force poorly correlated with level of mechanical restoration. Direct measures of the disc, volume injected and change in disc height from injection, had the strongest relationship to mechanical restoration.

Conclusion: This work showed that measuring the injection force for injectable treatments of the disc can provide lower and upper limits for delivery, but direct measures are stronger indicators of disc mechanical restoration.

Keywords: Intervertebral disc degeneration, nucleus augmentation, surgical optimisation, biomaterials, biomechanics.

Statements and declarations:

Funding: Financial support through the grant EPSRC grant EP/R513258/1 is gratefully acknowledged.

Employment, Financial interests & Non-financial interests: Nothing to declare

Authors' contribution: Conceptualization (ARD, JPW, MM, RKW); Data curation (ARD, RKW); Formal Analysis (ARD, JPW, MM, RKW); Funding acquisition (RKW); Investigation (ARD, JPW, MPC); Methodology (ARD, JPW, MPC); Project administration (RKW); Supervision (MM, RKW); Visualization (ARD, JPW); Writing – original draft (ARD, JPW); Writing – review & editing (ARD, JPW, MPC, AK, MM, RKW). A. Dixon and J. Warren contributed equally to this paper.

1. Introduction

Nucleus augmentation, in which a biomaterial is injected into the intervertebral disc without prior denucleation, has been proposed as an early stage intervention for disc degeneration [1]. A number of injectable biomaterials are under development for nucleus augmentation, and studies have reported on both their biological and mechanical characteristics [2]. The biomechanical performance of intervertebral disc specimens augmented with candidate biomaterials has also been evaluated through a range of *in vitro* tests in cadaveric human and animal tissue, with some evidence of the ability of the treatment to restore short-term biomechanical function [3]. In these studies, the amount of biomaterial injected in the nucleus augmentation procedure has been either to a fixed volume or based on the haptic judgement of the researcher [2], and there has been little investigation to date on the effects of clinical variables such as the volume of injected material or the force required to deliver it.

In a clinical setting, if the volume of biomaterial injected into the nucleus were too low, then there would be less restoration of disc height or biomechanical function, while too great a volume could potentially increase the risk of herniation or end-plate infraction [4]. However, the optimum volume to inject would likely be governed by the size, extracellular constituents, and degenerative state of the disc. An alternative may be to measure the mechanical resistance of the disc to filling, by monitoring the force required to deliver the biomaterial [5].

The aim of this contribution was to investigate the relationship between clinically quantifiable measures of biomaterial delivery in nucleus augmentation, and the resulting mechanical performance of the augmented intervertebral disc. The study was conducted using an *in vitro* biomechanical model to allow the greatest control of confounding factors, and a previously developed peptide-glycosaminoglycan (GAG) injectable hydrogel [6, 7].

2. Materials and methods

2.1. Specimen preparation

Bovine tails (C1-C4) (n=12) were imaged and sectioned into bone-disc-bone units (BDBU) retaining a consistent thickness of vertebral bone [8]. The exposed bone was cleaned using a surgical water pik (Pulsavac Plus Wound Debridement System, Zimmer Biomet, USA) and the specimens were placed in an agitated anticoagulant bath at 4 °C for 24 hours (sodium citrate, 20.5 mM). Specimens were rinsed and frozen to -80 °C until the day of testing.

Commented [MM1]: I think we should say how many tails were used

2.2. Overview

The prepared BDBUs were subjected to a sequential testing protocol, where the units were biomechanically tested in the native state, subjected to a rapid enzymatic degeneration procedure, tested in the artificially degenerated state, injected with a hydrogel, and finally tested in the treated state. A summary of the testing is shown in Figure 1A.

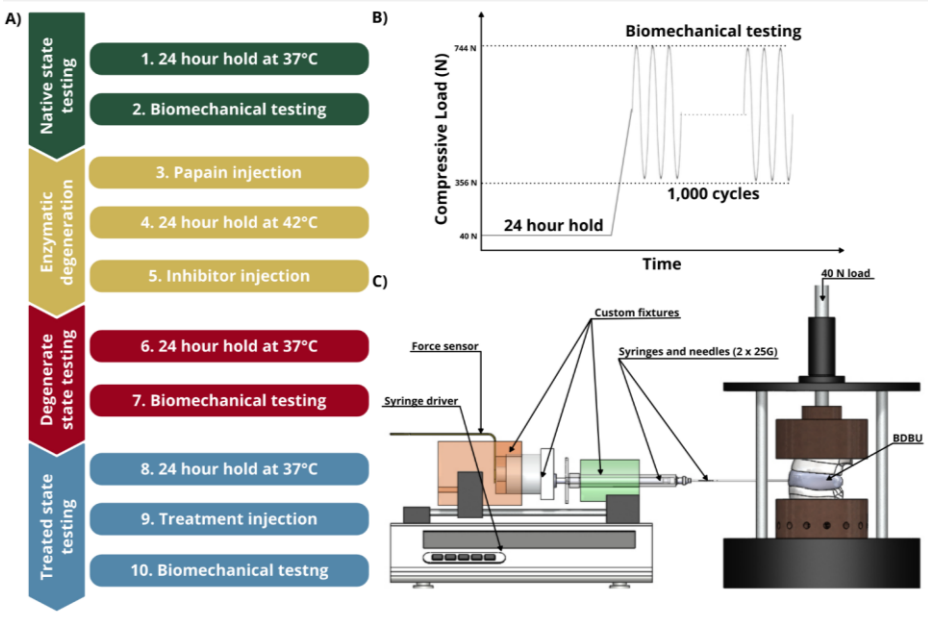

Figure 1 – A) Summary flow chart of test procedure, B) hold period and biomechanical test compressive loading, and C) treatment injection fixture set up (not to scale).

2.3. Biomechanical testing

BDBUs were biomechanically tested in the native, degenerate and treated states. To establish a physiologically relevant osmotic equilibrium, and ensure consistency of hydration between tests, the

BDBM units were held for a 24 hour period in a phosphate buffer saline (PBS) bath under a 40 N load prior to each testing phase (steps 1, 6, and 8 in Figure 1A). After the hold period, the BDBUs were subjected to a 1,000 cycle cyclic compression test at 1 Hz between 300 N and 740 N as shown in Figure 1B (steps 2, 7, and 10). Cyclic compression testing was conducted within a PBS bath using an electromechanical testing machine (ElectroPuls E10000, 2527 Series 10 kN Bi-axial Dynacell, Instron, USA). After each cyclic compression test, specimens were imaged using micro-computed tomography (74 µm voxel size, µCT100, Scanco Medical AG, Switzerland).

2.4. Enzymatic degeneration

After the native state testing, the BDBUs were artificially degenerated by injecting 0.3 ml papain (1.6 kU/ml) with a 30G needle to non-selectively break down the collagen and proteoglycan structures within the nucleus [9]. The units were then left to digest for a 24 hour hold period under 40 N load at 42 °C (step 4) and then 0.3 ml of a papain inhibitor, ebselen (0.064 µmol), was injected preventing further degeneration (step 5). Following biomechanical testing (steps 6 and 7), the units were frozen to -80 °C until ready for treatment.

2.5. Nucleus augmentation

The nucleus augmentation procedure was carried out between the hold period and the cyclic compression test (Step 9). The augmentation was performed using a peptide-GAG hybrid hydrogel (P_{11}-12 and chondroitin sulfate, 1:20 ratio [6, 7]). Lyophilised peptide (P_{11}-12, Ac-SSRFOWOFESS-NH_2) was weighed out (20 mg, 0.014 mol, 95% purity, CS Bio, CHEMGO Organica AG, Switzerland) into glass vials and reconstituted in 130mM NaCl aqueous solution (500 µl). Lyophilised chondroitin sulfate (CS) (MW ~ 50 kDa, ZPD, Zeria Pharmaceutical Co., Ltd, Denmark) was weighed out (137 mg) into glass vials and reconstituted in 130mM NaCl aqueous solution (500 µl). The samples were then vortexed (30s) at 2500 rpm on a vortex mixer. After early analysis of the augmented discs, an additional step was added to sonicate the peptide and GAG components to reduce the likelihood of microbubble formation. The peptide and GAG components were injected separately via two 25G needles into the centre of the nucleus pulposus, such that mixing and gelation occurred *in situ*. To allow radiographic analysis of the injected material in the post-test CT scans, a radio-opaque agent (Ultravist®) and UV fluorescent dye (5,6-carboxyfluorescein) were added to the gel components. The injection was performed using a custom rig that connected two syringes in parallel to a syringe driver such that both components were delivered at a constant rate. A 40 N axial compressive load was applied to the BDBU during injection, as shown in Figure 1C. A transducer (Flexiforce B201 series, Tekscan Inc, USA) was inserted between the syringe driver and the rear of the custom injection rig to measure the force applied to the syringes ('injection force'). The volume of hydrogel

injected was measured using the syringe markings. Injection was stopped when a predetermined load was reached, the syringes were empty, or if the syringe driver began to slip repeatedly. The pre- and post-injection BDBU height were recorded using Vernier callipers.

2.6. Data analysis

Specimens were excluded from the analysis if they presented less than 10% change in stiffness following the degeneration procedure, or if the post-test radiographic analysis revealed that either the injections were not into the nucleus, or there were observable coalesced air microbubbles. A total of 22 specimens were analysed. Using an ad-hoc script (MATLAB and Statistics Toolbox Release 2021a, The MathWorks, Inc., Natick, Massachusetts, United States), the stiffness of each individual loading cycle was computed from a linear fit of the load-displacement data, excluding 5 points of the extreme values (corresponding to approximately 10% of the cycle at the beginning and the end of the loading phase). From each 1,000 cycles of testing for each BDBU state, the mean stiffness over the last ten cycles was extracted for comparison between samples and states (hereafter referred to simply as "stiffness"). Mechanical restoration following treatment was defined as the difference between the native and treated stiffness.

Due to the paired and non-normal nature of the data, a Friedman test was applied to compare the different states, with post hoc Wilcoxon sign rank tests (α=0.05). All specimens were placed into two sets of groupings related to the total volume injected (low < 0.85 ml, 0.85 ml < medium < 1.25 ml, high > 1.25ml) and to the maximum injection force achieved recorded during the delivery (low <30 N, 30 N < medium < 40 N, high > 40 N). Due to the small sample sizes in the subgroups, and the nature of the data, only the median, maximum and minimum values for these subgroups were investigated. Statistical analysis was performed using the Statistics Toolbox in MATLAB (v 2021a).

The mechanical restoration was evaluated against the six measurable or calculable in clinic parameters using linear regressions: volume injected, volume injected/disc cross-sectional area, injection force, injection force/disc cross-sectional area, work done (integral of injection force-time plot), and change in BDBU height following injection (post-injection height minus pre-injection height).

All data is in this study is available open access at the Leeds data repository [10].

Commented [MM2]: Add reference:
Dixon, Warren, Mengoni and Wilcox, 2023, dataset: Bovine intervertebral disc longitudinal in vitro study: biomechanical effect of volume and pressure of hydrogel injection into intervertebral discs

3. Results

In all specimens and states, the stiffness increased throughout the duration of the cyclic loading and the stiffness in the degenerated state was higher than in the native state for each specimen. Typical profiles for the stiffness of a specimen across the three states are shown in Figure 2A; the stiffness for all specimens in each state is shown in Figure 2B. During the nucleus augmentation procedure, there was an initial ramp in the injection force, followed by a period where force remained relatively steady before it rose more steeply. An example showing these three phases is shown in Figure 2C.

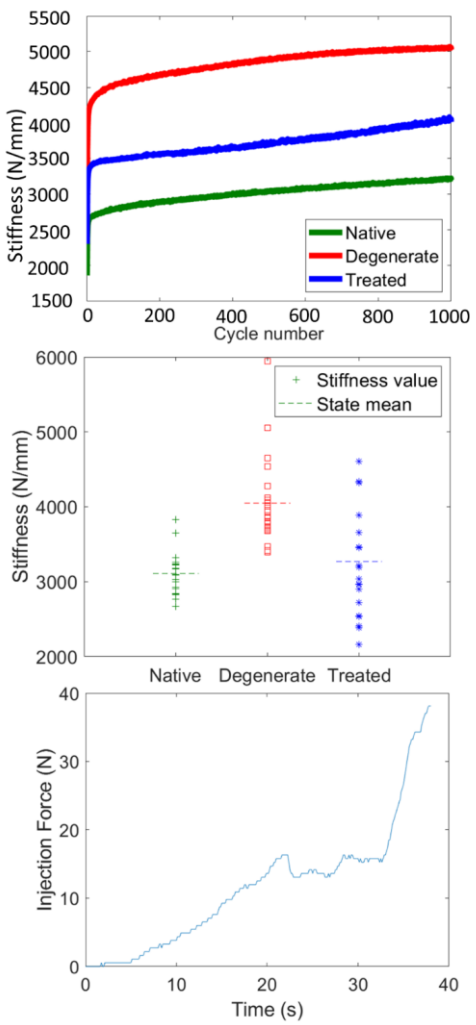

Figure 2 – A) Typical stiffness plot for different states, and B) Comparison of stiffness in different states for all specimen C) typical injection force plot

Significant differences were found in stiffness values between the degenerate and the native states (post-hoc Wilcoxon test, $p<0.001$) or between the degenerate and the treated states (post-hoc Wilcoxon test, $p<0.001$), and no difference between the treated and native states (post-hoc Wilcoxon test, $p=0.78$). The median (max, min) of the stiffness restoration for the low, medium, and high volume groups were: - 1.24 (-0.38, -1.92) kN/mm, 0.19 (0.70, -0.36) kN/mm, and 0.28 (0.75, - 0.14) kN/mm respectively, a summary plot of the stiffness restoration is shown in Figure 3A. Similarly, the low, medium and high force groups median (max, min) restoration values were: -0.36 (0.50, -1.92) kN/mm, -0.09 (0.70, -1.43) kN/mm, and 0.12 (0.74, -0.43) kN/mm respectively. Mechanical restoration was compared against parameters of interest, an example is shown in Figure 3B. The r-squared and p values of linear regressions for all parameters are shown in Table 1.

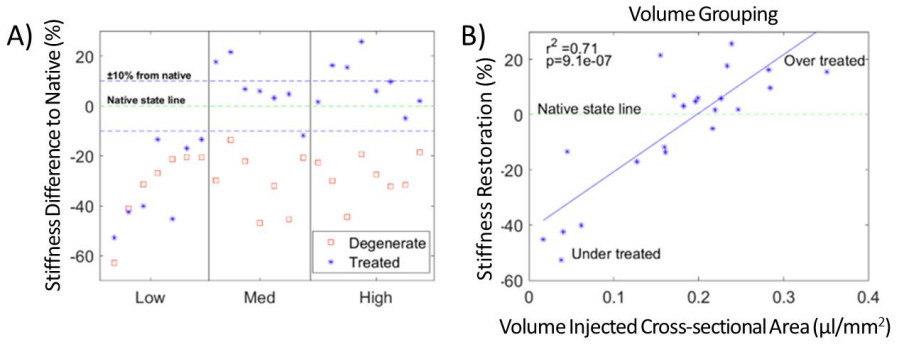

Figure 3 - A) Summary of stiffness differences compared to native state for low, medium, and high volume specimens, B) Linear regression relationship between mechanical restoration and volume/cross sectional.

Table 1 – Relationship (linear regression) between parameters and treatment stiffness mechanical restoration

Parameter	R-squared	p-value
Volume injected (ml)	0.63	< 0.001
Volume injected/Cross sectional area (µl/mm^2)	0.71	< 0.001
Max Injection force (N)	0.23	0.023
Max Injection force/cross sectional area (N/mm^2)	0.28	0.011
Work done (J)	0.31	0.007
Change in height (pre injection – post injection) (mm)	0.79	< 0.001

4. Discussion

The objective of this work was to assess the use of clinically relevant quantitative measures for nucleus augmentation. Three key variables were measured: the volume injected, the injection force, and the change in disc height following augmentation. The results showed that the nucleus augmentation procedure was able to restore specimen stiffness, with no statistically significant difference found across all the data between the native and treated states. When divided into the subsets based on the volume injected or the applied force, a minimum volume was required to achieve mechanical restoration (Figure 3A).

Commented [MM3]: These 2 figures are not vertically aligned which I find very unpleasant to look at

Commented [MM4R3]: + may be better side by side than on top of each other

The hydrogel was able to reduce the stiffness of the disc from the degenerate state to that of the native state, with no significant difference. However, the degenerate model used has some limitations as it is not possible to mimic all stages of degeneration in the lab. Some variation in the severity of degeneration were observed due to natural tissue variation, however, the degenerate model was only representative of an early-stage degeneration level, with degeneration induced from the enzyme localised to the nucleus. This may not be fully representative of degeneration where annular tears can be observed clinically [17, 18]. Instead, the degenerate model was representative of degeneration with minimal annular pathology [19] which would be a target degeneration level for a nucleus augmentation treatment.

Previous *in vitro* studies of nucleus augmentation had stopped injection based either on reaching a given volume [11–13] or haptic feedback [14–16]. Not only was the injection force dependent on the state of the disc but also the fluid viscosity, the syringe, and the needle gauge and length which these previous studies made reference to, but the effects of systematically varying either volume or injection force were not assessed as reasons for stopping the injection. In this study, it was found that the volume of biomaterial injected, the volume normalised by disc cross-sectional area, and change in disc height from injection, provided good indicators of the level of mechanical restoration of disc biomechanical properties. The strongest relationship identified was with the change in disc height pre- and post- injection, which is unsurprising given the relationship between height and stiffness. Overall, the relationship between the direct volume and height measurements and the level of stiffness restoration was much better than for the measurements derived from the applied injection force.

Clinically, the volume and height measurements may be readily implemented. Volume can be monitored on syringes and change in disc height may be approximated with fluoroscopy or other available imaging modality during surgery. The volume of injected biomaterial normalised by the disc cross-sectional area was better correlated to the mechanical restoration than the volume alone. The curved nature of the endplates meant the consistent measures of disc height were more difficult to derive from the CT data, so only the disc cross-sectional area rather than the disc volume was used to normalise the injection volume, and the stiffness was not normalised (i.e. as an apparent modulus) to the disc dimensions. Clinically, there could be potential to better estimate all of the disc dimensions from additional magnetic resonance imaging [20].

The injection force was monitored as a parameter to provide a quantitative measure of the haptic feedback used clinically to determine intradiscal pressure. As the hydrogel is injected into the disc, the expansion of the nucleus will be resisted by the tensioning of the annulus tissues and the intradiscal pressure will increase. The force required to inject the biomaterial was therefore expected to increase

during the delivery [21, 22]. This study found that the injection force initially increased to a steady value, which was maintained for a period of time before rising steeply. In the *in vitro* model used here, the artificial degeneration of the disc caused a loss of disc height and increased stiffness during mechanical testing, where our current hypothesis is the degeneration potentially caused a loss of GAG and fluid content in the nucleus resulting in the altered biomechanics. There is therefore little resistance to the injection initially as the disc 'refills'. It is only once the annulus becomes tensioned that the force increases consistently. Whilst the maximum injection force would in theory be a good indicator of fill, in this study all measures associated with injection force poorly correlated with the mechanical restoration of biomechanical function. It is likely that *in vivo*, the progressive degeneration of the disc and remodelling of the surrounding tissues would provide greater resistance to injection in the initial stages, and the injection force would be more difficult to interpret. Therefore, injection force alone does not appear to provide sufficient data to be used as a clinical measurement tool during nucleus augmentation. Nevertheless, when used in conjunction with the directly measured volume and height parameters, injection force could still provide a meaningful upper limit measurement to prevent damage. An alternative to measuring the injection force would be to directly monitor the nuclear pressure; however, this is associated to a range of issues and constraints [23-25].

In this study, the biomaterial used for the nucleus augmentation was a previously developed peptide-GAG hybrid hydrogel [6, 7]. The GAG component acts both to trigger rapid self-assembly of the peptide, and also to mimic the healthy nucleus tissue's ability to imbibe water. By having a similar GAG composition to the native nucleus, and a hydrogel with a high water content, the aim was that the natural balance between the load and osmotic pressure would reduce risks associated with overfilling. It should, therefore, be noted that while the results of this study are applicable to other nucleus augmentation materials, the ranges of the measured injection parameters likely depend on the biomaterial used and its properties. It is likely that materials with different osmotic potential, gelation mechanisms or mechanical properties could result in different biomechanical outcomes for a given injected volume. The needle gauge and length used for injection will also affect the applied forces. Although the relationships between injection parameters and resultant biomechanical performance may be unique to a given material, the parameters and techniques developed in this work could readily be applied to other biomaterials.

5. Conclusions

This research clearly demonstrates that mechanical restorative outcomes for nucleus augmentation vary with injection parameters. Specifically, the maximum force required to inject the hydrogel has a weak relationship with mechanical restoration but could provide upper and lower limits for injection.

242 Whereas the volume injected and change in height from injection have strong relationships with
243 mechanical restoration and are likely suitable outcome predictors.

244

References

1. Iatridis JC, Nicoll SB, Michalek AJ, et al (2013) Role of biomechanics in intervertebral disc degeneration and regenerative therapies: What needs repairing in the disc and what are promising biomaterials for its repair? Spine J 13:243–262. https://doi.org/10.1016/j.spinee.2012.12.002

2. Schmitz TC, Salzer E, Crispim JF, et al (2020) Characterization of biomaterials intended for use in the nucleus pulposus of degenerated intervertebral discs. Acta Biomater. 114:1–15

3. Dixon AR, Warren JP, Culbert MP, et al (2021) Review of in vitro mechanical testing for intervertebral disc injectable biomaterials. J Mech Behav Biomed Mater 123:104703. https://doi.org/10.1016/J.JMBBM.2021.104703

4. Hebelka H, Nilsson A, Hansson T (2014) Pressure increase in adjacent discs during clinical discography questions the methods validity. Spine (Phila Pa 1976). https://doi.org/10.1097/BRS.0000000000000166

5. Robinson TE, Hughes EAB, Bose A, et al (2020) Filling the Gap: A Correlation between Objective and Subjective Measures of Injectability. Adv Healthc Mater. https://doi.org/10.1002/adhm.201901521

6. Miles DE, Mitchell EA, Kapur N, et al (2016) Peptide:glycosaminoglycan hybrid hydrogels as an injectable intervention for spinal disc degeneration. J Mater Chem B 4:3225–3231. https://doi.org/10.1039/c6tb00121a

7. Warren JP, Miles DE, Kapur N, et al (2021) Hydrodynamic Mixing Tunes the Stiffness of Proteoglycan-Mimicking Physical Hydrogels. Adv Healthc Mater. https://doi.org/10.1002/adhm.202001998

8. Sikora SNF, Miles DE, Tarsuslugil S, et al (2018) Examination of an in vitro methodology to evaluate the biomechanical performance of nucleus augmentation in axial compression. Proc Inst Mech Eng Part H J Eng Med 232:230–240. https://doi.org/10.1177/0954411917752027

9. Amri E, Mamboya F (2012) Papain, a plant enzyme of biological importance: A review. Am. J. Biochem. Biotechnol. 8:99–104

10. Dixon AR, Warren JP, Culbert MP, Khan A, Mengoni M, Wilcox RK, 2023, dataset: Bovine intervertebral disc longitudinal in vitro study: biomechanical effect of volume and pressure of hydrogel injection into intervertebral discs, Leeds Data Repository: https://archive.researchdata.leeds.ac.uk/

11. Khalaf K, Nikkhoo M, Kuo YW, et al (2015) Recovering the mechanical properties of denatured intervertebral discs through Platelet-Rich Plasma therapy. In: Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBS. pp 933–936

12. Leckie AE, Akens MK, Woodhouse KA, et al (2012) Evaluation of thiol-modified hyaluronan and elastin-like polypeptide composite augmentation in early-stage disc degeneration: Comparing 2 minimally invasive techniques. Spine (Phila Pa 1976) 37:. https://doi.org/10.1097/BRS.0b013e318266ecea

13. Saunders JM, Tong T, Le Maitre CL, et al (2007) A study of pH-responsive microgel dispersions: From fluid-to-gel transitions to mechanical property restoration for load-bearing tissue. Soft Matter 3:486–494. https://doi.org/10.1039/b613943d

14. Balkovec C, Vernengo J, McGill SM (2013) The use of a novel injectable hydrogel nucleus

pulposus replacement in restoring the mechanical properties of cyclically fatigued porcine intervertebral discs. J Biomech Eng 135:061004. https://doi.org/10.1115/1.4024285

15. Pelletier MH, Cohen CS, Ducheyne P, Walsh WR (2016) Restoring segmental biomechanics through nucleus augmentation. Clin Spine Surg 29:461–467. https://doi.org/10.1097/BSD.0b013e3182aa6841

16. Thorpe AA, Boyes VL, Sammon C, Le Maitre CL (2016) Thermally triggered injectable hydrogel, which induces mesenchymal stem cell differentiation to nucleus pulposus cells: Potential for regeneration of the intervertebral disc. Acta Biomater 36:99–111. https://doi.org/10.1016/j.actbio.2016.03.029

17. Sharma A, Pilgram T, Wippold FJ (2009) Association between Annular Tears and Disk Degeneration: A Longitudinal Study. AJNR Am J Neuroradiol 30:500. https://doi.org/10.3174/AJNR.A1411

18. Videman T, Nurminen M The Occurrence of Anular Tears and Their Relation to Lifetime Back Pain History: A Cadaveric Study Using Barium Sulfate Discography. Spine (Phila Pa 1976) 29:2668–2676

19. Jin L, Balian G, Li XJ (2018) Animal Models for Disc Degeneration-an Update. Histol Histopathol 33:543. https://doi.org/10.14670/HH-11-910

20. Deneuville JP, Yushchenko M, Vendeuvre T, et al (2021) Quantitative MRI to Characterize the Nucleus Pulposus Morphological and Biomechanical Variation According to Sagittal Bending Load and Radial Fissure, an ex vivo Ovine Specimen Proof-of-Concept Study. Front Bioeng Biotechnol. https://doi.org/10.3389/fbioe.2021.676003

21. Panjabi M, Brown M, Lindahl S, et al (1988) Intrinsic disc pressure as a measure of integrity of the lumbar spine. Spine (Phila Pa 1976) 13:913–917. https://doi.org/10.1097/00007632-198808000-00008

22. Cannella M, Isaacs JL, Allen S, et al (2014) Nucleus implantation: The biomechanics of augmentation versus replacement with varying degrees of nucleotomy. J Biomech Eng 136:051001. https://doi.org/10.1115/1.4027056

23. Elliott DM, Yerramalli CS, Beckstein JC, et al (2008) The effect of relative needle diameter in puncture and sham injection animal models of degeneration. Spine (Phila Pa 1976) 33:588–596. https://doi.org/10.1097/BRS.0b013e318166e0a2

24. Wang JY, Mansfield JC, Brasselet S, et al (2021) Micro-mechanical damage of needle puncture on bovine annulus fibrosus fibrils studied using polarization-resolved Second Harmonic Generation(P-SHG) microscopy. J Mech Behav Biomed Mater. https://doi.org/10.1016/j.jmbbm.2021.104458

25. McNally DS, Adams MA (1992) Internal intervertebral disc mechanics as revealed by stress profilometry. Spine (Phila Pa 1976) 17:66–73. https://doi.org/10.1097/00007632-199201000-00011