

Thermally Triggered Hydrogel Injection Into Bovine Intervertebral Disc Tissue Explants Induces Differentiation Of Mesenchymal Stem Cells And Restores Mechanical Function.

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1 **Thermally Triggered Hydrogel Injection Into Bovine Intervertebral Disc Tissue**
2 **Explants Induces Differentiation Of Mesenchymal Stem Cells And Restores**
3 **Mechanical Function.**

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23

24

25 **Abstract**

26 We previously reported a synthetic Laponite® crosslinked pNIPAM-co-DMAc (L-
27 pNIPAM-co-DMAc) hydrogel which promotes differentiation of mesenchymal stem
28 cells (MSCs) to nucleus pulposus (NP) cells without additional growth factors. The
29 clinical success of this hydrogel is dependent on: integration with surrounding tissue;
30 the capacity to restore mechanical function; as well as supporting the viability and
31 differentiation of delivered MSCs. Bovine NP tissue explants were injected with
32 media (control), human MSCs (hMSCs) alone, acellular L-pNIPAM-co-DMAc
33 hydrogel or hMSCs incorporated within the L-pNIPAM-co-DMAc hydrogel and
34 maintained at 5% O₂ for 6 weeks. Viability of native NP cells and delivered MSCs
35 was maintained. Furthermore hMSCs delivered via the L-pNIPAM-co-DMAc hydrogel
36 differentiated and produced NP matrix components: aggrecan, collagen type II and
37 chondroitin sulphate, with integration of the hydrogel with native NP tissue. In
38 addition L-pNIPAM-co-DMAc hydrogel injected into collagenase digested bovine
39 discs filled micro and macro fissures, were maintained within the disc during loading
40 and restored IVD stiffness. The mechanical support of the L-pNIPAM-co-DMAc
41 hydrogel, to restore disc height, could provide immediate symptomatic pain relief,
42 whilst the delivery of MSCs over time regenerates the NP extracellular matrix; thus
43 the L-pNIPAM-co-DMAc hydrogel could provide a combined cellular and mechanical
44 repair approach.

45 **Abbreviations**

46 LBP: low back pain; IVD: Intervertebral disc; CEP: cartilaginous end plate; AF:
47 annulus fibrosus; NP: nucleus pulposus; ECM: extracellular matrix; MSCs:
48 mesenchymal stem cells; hMSCs: human mesenchymal stem cells; L-pNIPAM-co-

49 DMAc: Laponite® crosslinked pNIPAM-co-DMAc hydrogel; FCS: fetal calf serum,
50 NIPAM: N-isopropylacrylamide; DMAc: N, N' –dimethylacrylamide; AIBN: 2-2'-
51 azobisisobutyronitrile; SEM: scanning electron microscopy; IHC:
52 Immunohistochemistry.

53 **Keywords**

54 Intervertebral disc, Nucleus pulposus, Injectable hydrogel, Mesenchymal stem cells,
55 Tissue explant culture.

56 **1. Introduction**

57 Low back pain (LBP) is an increasingly prevalent clinical condition that affects over
58 80% of the population at some point during their lifetime [1]. The aetiology of chronic
59 LBP is thought to be multifactorial; however, degeneration of the intervertebral disc
60 (IVD) is regarded as a key attributing factor [2,3]. Morphologically the IVD can be
61 divided into three distinct regional structures: the cartilaginous endplates (CEP); the
62 annulus fibrosus (AF) and the central gelatinous nucleus pulposus (NP), rich in
63 proteoglycans (mainly aggrecan) and collagen type II [4]. The IVD transmits load,
64 facilitates a range of spinal movement and dissipates energy during motion [5].

65 Degeneration of the IVD is characterised by a number of progressive extracellular
66 matrix (ECM) changes including altered matrix synthesis and increased degradation
67 of normal matrix components [6], resulting in an overall reduction in the proteoglycan
68 content of the NP [7]. This is mediated by the catabolic phenotype of degenerate NP
69 cells [8,9] alongside decreased viability as well as increased apoptosis [17] and
70 senescence of remaining NP cells [10]. This results in reduced NP tissue hydration
71 and an overall loss in disc height [11], culminating in mechanical failure resulting in

72 abnormal stresses to surrounding spinal tissues and compression of nerve routes
73 [12]. Consequently, the restoration of disc height is a key therapeutic target for the
74 symptomatic relief of chronic LBP.

75 New approaches in tissue engineering have led to the investigation of a variety of
76 treatment options aimed at the restoration of disc height using acellular implants [13-
77 16], the use of regenerative cells [17-20] or the combined delivery of regenerative
78 cells with supporting mechanical scaffolds [21,22]. Mesenchymal stem cells (MSCs)
79 have been highlighted as an attractive cell choice since they have proliferative
80 capacity, can be extracted from a variety of adult tissues and have the capacity to
81 differentiate into NP like cells [19,20,23-25]. However, concerns remain regarding
82 MSC leakage [26], as well as a lack of control over differentiation following injection
83 [27], thus highlighting the potential need for cell carrier systems. The use of a
84 combined approach is appealing, since it is hypothesised that the mechanical
85 support of the biomaterial scaffold would provide immediate pain relief. Whilst the
86 delivery of regenerative cells would provide a long term, gradual regeneration of an
87 ECM which biologically functions, akin to native NP tissue. To date, a variety of
88 biomaterial scaffolds have been investigated for repair of the NP. However injectable
89 scaffolds with the potential to deliver cells, have not demonstrated the required
90 mechanical robustness [14]. Whilst those developed as acellular NP replacement
91 scaffolds have so far failed, due to poor integration resulting in extrusion or expulsion
92 of the implanted material [14,28,29] or are unable to deliver regenerative cells to the
93 IVD [13-16]. The implantation methodology is also an important consideration to
94 minimise trauma to the existing IVD tissue [30,31].

95 We have previously reported the development of a synthetic Laponite® crosslinked
96 pNIPAM-co-DMAc (L-pNIPAM-co-DMAc) hydrogel delivery system which has the
97 potential to deliver human MSCs (hMSCs) via minimally invasive injection, using
98 small bore needles (26G) which decrease the chance of inducing damage to the
99 annulus fibrosus [23]. We have demonstrated *in vitro* that hMSCs incorporated into
100 L-pNIPAM-co-DMAc hydrogels and cultured in 5% O₂, differentiated into NP-like
101 cells, without the use of chondrogenic inducing medium or additional growth factors
102 [23]. The clinical success of this hydrogel is dependent on: integration with
103 surrounding tissue; the capacity to restore mechanical function; as well as supporting
104 the viability and differentiation of delivered MSCs.

105 In the present study, we investigated the efficacy of several IVD repair strategies
106 including: hMSCs alone, acellular L-pNIPAM-co-DMAc hydrogel or hMSCs
107 incorporated within L-pNIPAM-co-DMAc hydrogel, injected into bovine NP tissue
108 explants. This study tested the hypothesis that the delivery of hMSCs within the L-
109 pNIPAM-co-DMAc hydrogel would aid scaffold integration, and promote
110 differentiation of MSCs towards the correct NP cell phenotype within native NP
111 tissue. We additionally investigated the mechanical function, defined in this study as
112 disc apparent modulus, strain under load, energy dissipation, and restoration of
113 hydrogel injected bovine IVDs following collagenase digestion. Together these
114 investigations determine the capacity of this hydrogel to be used as both a cell
115 delivery vehicle and as a mechanical support scaffold in the treatment of IVD
116 degeneration.

117 **2. Materials and Methods**

118 **2.1. Nucleus pulposus tissue explant culture**

119 Bovine tails from 9 months old to 18 months old cows were obtained from the
120 abattoir. Caudal IVDs were excised and NP tissue was isolated. Cores of NP tissue
121 (0.5cm in diameter) were formed and placed in a Perspex® ring, in sterile 6 well
122 culture plates, as previously described [17]. Ten millilitres of DMEM media (Life
123 Technologies, Paisley UK) supplemented with 10% v/v heat inactivated foetal calf
124 serum (FCS) (Life Technologies, Paisley UK), 100U/ml penicillin (Life Technologies
125 Paisley UK), 100µg/ml streptomycin (Life Technologies Paisley UK), 250ng/ml
126 amphotericin (Sigma, Poole UK), 2mM glutamine (Life Technologies, Paisley UK)
127 and 10µg/ml ascorbic acid (Sigma, Poole UK) (complete cell culture media) was
128 applied and tissue explants and were maintained in culture for 48 hours prior to
129 hMSC and hydrogel injection.

130 **2.2. Hydrogel synthesis**

131 An exfoliated suspension of Laponite® clay nanoparticles (25-30nm diameter, <1nm
132 thickness) (BYK Additives Ltd, Cheshire UK) was prepared by vigorous stirring of
133 Laponite® (0.1g) in deionised H₂O (10ml) (18 MΩ) for 24 hours. N-
134 isopropylacrylamide 99% (NIPAM) (0.783g) (Sigma, Poole UK), N, N' -
135 dimethylacrylamide (DMAc) (0.117g) (Sigma, Gillingham UK) and 2-2'-
136 azobisisobutyronitrile (AIBN) (0.009g) (Sigma, Poole UK) were added to the
137 suspension and stirred for 1 hour. After passing the suspension through a 5-8µm
138 pore filter paper, polymerisation was initiated by heating to 80°C and the reagents
139 were allowed to react for 24 hours. Following 24 hours the hydrogel suspension was
140 cooled to 38-39°C prior to cell incorporation. Further cooling of the polymeric
141 suspension to 37°C, i.e. below the lower critical solution temperature (LCST),
142 resulted in rapid gelation to a solidified hydrogel, as previously described [23].

143

144 **2.3. Mesenchymal stem cell source, expansion and transfer to nucleus**
145 **pulposus tissue explants.**

146 Commercial bone marrow derived human adult mesenchymal stem cells (hMSCs)
147 extracted from a 39yr old donor (Lonza, Slough UK) were cultured in complete
148 DMEM media (Life Technologies, Paisley UK). MSCs were expanded in monolayer
149 culture to passage 7 to ensure sufficient cells were available. To allow cell tracking
150 following injection, the MSCs were labelled with a fluorescent intracellular
151 carboxyfluorescein N-hydroxysuccinimidyl ester (CFSE) cell labelling kit, (Abcam,
152 Cambridge, UK) according to manufacturer's instructions. Cell seeding solutions
153 were prepared at a density of 4×10^6 cells/ml in either complete DMEM media or
154 liquid hydrogel suspension (38-39°C) and then 50µl injected into the centre of NP
155 tissue explants via 26 gauge needle injection (Becton Dickinson, Plymouth, UK).
156 Fifty microliters of media or hydrogel suspension containing no cells was also
157 injected via a 26 gauge needle (Becton Dickinson, Plymouth, UK) into NP tissue
158 explants. This gave four experimental groups: media injected control (control), hMSC
159 injected alone (hMSC), acellular L-pNIPAM-co-DMAc hydrogel (acellular Hy) and
160 hMSC incorporated within L-pNIPAM-co-DMAc hydrogel (hMSC + Hy) injected NP
161 tissue explants. All NP tissue explants were cultured in complete DMEM cell culture
162 media via careful overlay of 10ml of media per NP tissue explant, incubated at 37°C,
163 5% CO₂ and maintained in culture for up to 6 weeks in an oxygen controlled glove
164 box (Coy Lab products, York, UK) at 5% O₂. Media was replaced every 2-3 days.
165 Samples were removed after 48 hours, 2, 4 and 6 weeks for analysis of cell viability,
166 scanning electron microscopy (SEM), histological assessment of matrix deposition
167 and NP cell phenotype analysis using immunohistochemistry (IHC).

168 **2.4. Cytospins**

169 IHC was performed on MSCs taken from monolayer culture prior to injection into NP
170 tissue explants to serve as time zero controls. Monolayer cells were trypsinised and
171 cells fixed in 4% w/v paraformaldehyde/PBS (Sigma, Poole UK) for 20min, spun at
172 300g for 5 min to form a cell pellet and resuspended in PBS to a cell density of 300
173 cells per microlitre. One hundred microlitres of cell suspension was then cytopun,
174 formed via centrifugation at 1000rpm for 3 min (Shandon Cytospin 3, Thermo
175 Scientific, Loughborough UK). Slides were subsequently air-dried and stored at 4°C
176 until required for IHC analysis.

177 **2.5. Processing of tissue explants and identification of the injection site:**

178 Triplicate NP tissue explants of each experimental group: control, hMSC alone,
179 acellular Hy and hMSC + Hy, were fixed in 10% w/v formalin (Leica Microsystems,
180 Milton Keynes UK) overnight prior to routine paraffin embedding. Tissue samples
181 were serially sectioned at 4µm, perpendicular to the needle injection, and two
182 sections every 100µm were mounted onto positively charged slides (Leica
183 Microsystems, Milton Keynes UK). Sections were air-dried, dewaxed in Sub-X (Leica
184 Microsystems, Milton Keynes UK), dehydrated in industrial methylated spirit (IMS)
185 (Fisher, Loughborough UK), washed in deionised H₂O and then stained with either
186 routine haematoxylin and eosin (H&E) (Leica Microsystems, Milton Keynes UK)
187 histological staining or Hoechst nuclear fluorescent staining (Sigma, Poole UK).
188 Slides stained for H&E were immersed in Mayers haematoxylin for 2 minutes, rinsed
189 in water for 5 minutes, immersed in eosin for 2 minutes, dehydrated in IMS, cleared
190 in Sub-X and mounted in Pertex® (Leica Microsystems, Milton Keynes UK). Slides
191 stained with Hoechst were immersed in 5pg/mL **Hoechst**/PBS, incubated for 10min,

192 washed 3 times in PBS and mounted in 90% v/v glycerol/PBS. Slides were then
193 visualised using light and fluorescent microscopy with an Olympus BX51 microscope
194 and images captured by digital camera and Capture Pro OEM v8.0 software (Media
195 Cybernetics, Buckinghamshire, UK) to identify the injection site and distinguish
196 native NP cells (Hoechst+/CFSE-) and injected hMSCs (Hoechst+/CFSE+). Within
197 control NP explants, the injection site was identified where the needle track could be
198 seen histologically (within early time points) or halfway through the entire depth of
199 the NP tissue explant. Where MSCs were injected alone injection site was
200 determined via the identification of CFSE positive cells. Where acellular L-pNIPAM-
201 co-DMAc or L-pNIPAM-co-DMAc with incorporated MSCs had been injected, the
202 injection site was determined by the histological identification of the hydrogel.
203 Following identification of the position of the injection site and the presence of CFSE
204 positive cells, serial sections in the area of the injection site were mounted onto
205 positively charged slides: for caspase 3 IHC to identify the presence of apoptotic
206 cells, histological assessment of matrix components using Alcian blue and Masson
207 trichrome as well as IHC for aggrecan, chondroitin sulphate and collagen type II to
208 assess phenotypic characteristics.

209 **2.6. Structural and Mechanical Characterisation**

210 **2.6.1. Scanning electron microscopy (SEM).**

211 Samples were removed from culture after 48 hours and 6 weeks, frozen at -80°C
212 and subsequently freeze dried using a FD-1A-50 freeze drier set to -53°C, 3.8×10^{-4}
213 mbar overnight. The sample was then fractured to expose the interior surface
214 morphology attached onto an aluminium stub and then using a Quorum Technology

215 150 Q TES system coated with gold (10µA sputter current for 180 seconds with a 2.7
216 tooling factor) for imaging.

217 The fractured surfaces were examined using a FEI NOVA nanoSEM 200 scanning
218 electron microscope (SEM). Secondary electron images were obtained using
219 accelerating voltage 5KV at various magnifications ranging from 1000x to 40,000x.

220 **2.6.2. Hydration degree**

221 To evaluate the hydration degree of NP tissue explants, samples were extracted
222 from culture in triplicate following 48 hours, 2, 4 and 6 weeks and the wet weight (M_0)
223 of the NP tissue explants was measured. Samples were then freeze dried using a
224 FD-1A-50 freeze drier set to -53 °C, 3.8×10^{-4} mbar overnight to obtain the dry
225 weight (M_t). The hydration degrees of the NP tissue explants were calculated using
226 eq1:

$$\text{Hydration degree} = \frac{(M_0 - M_t)}{M_0} \times 100$$

227 (1)

228 **2.6.3. Dynamic mechanical analysis**

229 The mechanical properties of NP tissue explants were characterised following 6
230 weeks in culture. Replicate samples were removed from culture, blotted and their
231 mechanical properties characterised by DMA. Samples were trimmed using a 5mm
232 circumference circular biopsy punch from the centre of NP tissue explants, all
233 sample heights were measured and recorded using digital callipers prior to
234 measurement. Triplicate samples were analysed using a PerkinElmer DMA8000

235 model under confined compression mode at 25 °C, applying a sinusoidal force with a
236 0.02mm displacement at 2.5Hz.

237 **2.7. Histological evaluation of matrix components**

238 Matrix deposition around the injection site of NP tissue explants was investigated
239 following 48 hours, 2, 4 and 6 weeks in triplicate. Sections were dewaxed in Sub-X,
240 dehydrated in IMS, washed in deionised H₂O and then stained using histological
241 stains: 1% w/v Alcian blue (pH 2.5) (Sigma Aldrich, Poole UK) in 3% v/v acetic acid
242 (Sigma Aldrich, Poole UK) for 5 minutes with 1% Neutral red w/v (Sigma Aldrich,
243 Poole UK) used as a counter stain for 2 minutes or Masson trichrome (Bio-Optica,
244 Miller & Miller (Chemicals) Ltd, Hainault UK) according to the manufacturer's
245 instructions. Sections were dehydrated in IMS, cleared in Sub-X and mounted in
246 Pertex® (Leica Microsystems, Milton Keynes UK). All slides were examined with an
247 Olympus BX51 microscope and images captured by digital camera and Capture Pro
248 OEM v8.0 software (Media Cybernetics, Buckinghamshire, UK). Histological sections
249 were analysed, features noted and images captured to document their histological
250 appearance.

251 **2.8. Immunohistochemistry assessment of apoptosis and phenotypic** 252 **characteristics.**

253 Caspase 3 as a marker of apoptosis and NP matrix markers: aggrecan, collagen
254 type II and chondroitin sulphate were selected for immunohistochemistry (IHC) to
255 assess the viability and phenotypic characteristics of NP tissue explants and
256 delivered hMSCs. IHC was performed as previously described [32]. Briefly, 4µm
257 paraffin sections were de-waxed, rehydrated and endogenous peroxidase-blocked
258 using hydrogen peroxide (Sigma, Aldrich Poole UK). After washing in tris-buffered

259 saline (TBS; 20 mM tris, 150 mM sodium chloride, pH 7.5) sections were subjected
 260 to antigen retrieval methods (Table I). Following TBS washing, nonspecific binding
 261 sites were blocked at room temperature for 90 minutes with 25% w/v serum (Abcam,
 262 Cambridge, UK) (Table I) in 1% w/v bovine serum albumin in TBS. Sections were
 263 incubated overnight at 4°C with the appropriate primary antibody (Table I). Negative
 264 controls in which mouse or rabbit IgGs (Abcam Cambridge UK) replaced the primary
 265 antibody at an equal protein concentration were used (Table I). After washing in
 266 TBS, sections were incubated in 1:500 biotinylated secondary antibody (Table I).
 267 Disclosure of secondary antibody binding was by the HRP-streptavidin biotin
 268 complex (30 minute incubation) (Vector Laboratories, Peterborough, UK), TBS
 269 washing, followed by application of 0.08% v/v hydrogen peroxide in 0.65 mg/ml 3,3'-
 270 diaminobenzidine tetrahydrochloride (Sigma Aldrich, Poole UK) in TBS (20 minute
 271 incubation). Sections were counterstained with Mayer's haematoxylin, dehydrated in
 272 IMS, cleared in Sub-X and mounted in Pertex®. All slides were visualised using an
 273 Olympus BX51 microscope and images captured by digital camera and Capture Pro
 274 OEM v8.0 software (Media Cybernetics, Buckinghamshire, UK). Evaluation of
 275 caspase 3 IHC staining was performed by counting immunopositive and
 276 immunonegative cells for each section and immunopositive cells expressed as a
 277 percentage of the total count. Images were captured for IHC staining of NP markers:
 278 aggrecan, collagen type II and chondroitin sulphate to qualitatively analyse the
 279 injection site, native disc cells/injected cells and surrounding matrix.

Target Antibody	Clonality	Optimal Dilution	Antigen Retrieval	Secondary Antibody	Serum Block
Caspase 3	Rabbit Polyclonal	1:400	None	Goat anti Rabbit	Goat
Aggrecan	Mouse Monoclonal	1:100	Heat	Rabbit anti mouse	Rabbit

Collagen II	type	Mouse Monoclonal	1:200	Enzyme	Rabbit anti mouse	Rabbit
Chondroitin Sulphate		Mouse Monoclonal	1:400	Enzyme	Rabbit anti mouse	Rabbit

280 **Table I:** Target antibodies used for IHC, their optimal concentrations and antigen
281 retrieval methods. Heat antigen retrieval consisted of 10-minute microwave
282 irradiation in 0.05M tris buffer, pH 9.5 pre-heated to 60°C. Enzyme antigen retrieval
283 consisted of 30-minute incubation in TBS; 20 mM tris, 150 mM sodium chloride,
284 46.8mM calcium chloride dihydrate pH 7.5, containing 0.01% w/v α -chymotrypsin
285 from bovine pancreas at 37°C.

286

287 **2.9 Mechanical characterisation of L-pNIPAM-co-DMAc hydrogel injected** 288 **whole bovine IVDs.**

289 **2.9.1. IVD Isolation and Collagenase Digestion**

290 Bovine tails from 9 to 18 month old cows were obtained from the abattoir, operating
291 in concordance with animal welfare regulations. Tails were stored at -20°C shortly
292 post sacrifice and tested a maximum of one month post storage. Tails were thawed
293 at 5°C for 24 hours prior to sample preparation. Discs were dissected whole from tail
294 sections between cd1-2 and cd4-5. Discs without parallel faces or with visible signs
295 of damage were discarded. The remaining discs were allowed to equilibrate to room
296 temperature for 12 hours before testing, between 2 and 4 discs were gained from
297 each tail section. Discs were stored in airtight sealed bags to prevent dehydration
298 during this time. Discs were randomly assigned to four test groups (n=10 per group):
299 healthy; sham injected; collagenase digested and collagenase digested following
300 hydrogel injected. Healthy discs were not experimentally manipulated following
301 excision, sham injected discs were stabbed with a 21-gauge needle to assess the
302 effect of needle insertion. Collagenase digested and hydrogel injected discs were

303 injected with 100-200µl of a 2mg/mL collagenase solution (Sigma, Poole, UK) in
304 distilled water (injection was performed until either the internal pressure in the disc
305 prevented any further injection or a maximum of 200µl had been injected) and
306 incubated for 2 hour at 37°C.

307 **2.9.2 Injection of L-pNIPAM-co-DMAc hydrogel in whole bovine IVDs**

308 Following collagenase digestion, discs to be injected with hydrogel were inspected
309 morphologically to ensure collagenase digestion had successfully induced void
310 formation. Hydrogel injected discs then received an injection of 50-200µL of acellular
311 L-pNIPAM-co-DMAc hydrogel, with an incorporated green food dye for visulisation.
312 Hydrogel injected discs were left 30 minutes post injection to allow the L-pNIPAM-co-
313 DMAc hydrogel to stabilize and mimic the operation time for the patient, prior to
314 testing. Injection protocol consisted of the syringe being depressed until either the
315 internal pressure in the disc prevented any further intake or a maximum of 200µl of
316 L-pNIPAM-co-DMAc hydrogel had been injected. All the needle tips were inserted
317 into the centre of the NP before injection of material. Visual inspection during
318 injection allowed needle position to be observed through the deformation of
319 surrounding material.

320 **2.9.3. Mechanical loading of IVDs**

321 Discs were loaded cyclically using a dynamic test rig incorporating a hydraulic piston
322 controlled by Wavematrix 1.8 test software and a Labtronic 8800 hydraulic controller
323 (Instron. Mass, USA). Discs were placed between two smooth parallel metal platens
324 without further constraint. Discs with non-parallel surfaces were rejected prior to
325 testing and no lateral movement was observed during testing (indicating that disc
326 surfaces were parallel and all deflection was in the axial direction). Discs were

327 subjected to a sinusoidal load between 0.53 and 0.65 MPa at 2 Hz to simulate
328 walking based on known values from the literature [51-53]. Each disc was ramped to
329 a preload at the midpoint between the upper and lower boundaries at a rate of 0.01
330 kN/s, immediately upon reaching the preload, cyclic loading was applied for 100
331 seconds, representative of a short period of activity typical of activities of daily living
332 (ADLs) in all but the most severely impaired persons.

333 Three measures of mechanical response were tracked by the data acquisition tools
334 connected to Wavematrix. Engineering stiffness of each disc was measured as the
335 change in force divided by the change in displacement from minimum ($F_{min} = 0.53$
336 MPa x disc cross-sectional area) to maximum ($F_{max} = 0.65$ MPa x disc cross-
337 sectional area) loading. Strain was measured as the displacement between those
338 points divided by the disc height at minimum loading. Lastly the energy dissipated by
339 discs during each loading/unloading cycle was calculated from the **hysteresis in the**
340 **load deflection data.**

341

342 **2.10. Data processing and statistical analysis**

343 All tests were performed at least in triplicate. Data was assessed for normality using
344 the Shapiro Wilks test and found to be non-parametric and hence statistical
345 comparisons were performed by Kruskal-Wallis with a pairwise comparisons
346 (Conover-Inman) post hoc test performed with statistical significance accepted at
347 $p \leq 0.05$. Pairwise comparisons were made as follows: between all-time points and
348 between the different experimental groups for caspase 3 immunopositivity; between
349 the different experimental groups for mechanical analysis; hMSC alone, Acellular Hy
350 and hMSC + Hy compared with media injected control NP explants for hydration

351 assessment at each given time point. Data was then presented on graphs; all
352 replicates have been shown with median value indicated to demonstrate clearly the
353 spread of replicates.

354

355 **3. Results**

356 **3.1. Identification of injected L-pNIPAM-co-DMAc hydrogel and mesenchymal** 357 **stem cells.**

358 No CFSE (green) positive cells were identified within media injected controls or
359 acellular hydrogel injected NP tissue explants (Fig 1). CFSE positive MSCs were
360 identified where MSCs had been injected alone, where they remained in clusters at
361 the vicinity of the injection site following 4 weeks in culture (Fig 1). Some CFSE
362 positive cells appeared to have migrated away from the injection site following 6
363 weeks, although many were still in close proximity to each other (Fig 1). Infiltrating
364 native NP cells stained with Hoechst were identified within the acellular L-pNIPAM-
365 co-DMAc following 4 and 6 weeks in culture (Fig 1). CFSE positive MSCs were
366 identified within the L-pNIPAM-co-DMAc hydrogel throughout the 6 week culture
367 duration where they had been incorporated prior to injection (Fig 1). CFSE positive
368 MSCs were also observed within the native NP tissue surrounding the hydrogel
369 region following 6 weeks in culture (Fig 1).

370

371 **3.2. Caspase 3 cell viability.**

372 Low levels of apoptosis was observed in media injected control NP explant tissue,
373 with no significant difference in the number of caspase 3 immunopositive cells
374 throughout the 6 week culture duration (Fig 2a,b). Where acellular L-pNIPAM-co-
375 DMAc hydrogel had been injected no significant difference in the number of caspase

376 3 immunopositive cells was observed between native NP cells within the surrounding
377 NP tissue compared with native NP cells infiltrated within the hydrogel (Fig 2a,b).
378 Low levels of apoptosis within MSCs injected alone, with no significant difference in
379 the number of immunopositive cells observed throughout the 6 week culture duration
380 (Fig 2a,b). Where MSCs were incorporated within L-pNIPAM-co-DMAc hydrogel
381 prior to injection, no significant difference in the number of caspase 3
382 immunopositive cells was observed between cells found within the surrounding
383 native NP tissue and cells found within the L-pNIPAM-co-DMAc hydrogel itself (Fig
384 2a,b). No significant difference in the number of caspase 3 immunopositive cells was
385 observed between the different experimental groups at any time point (Fig 2a,b)
386 ($P>0.05$).

387

388 **3.3. Structural and mechanical characterisation**

389 **3.3.1. Scanning electron microscopy**

390 The interior micro scale morphology of NP tissue explants was examined using
391 scanning electron microscopy (SEM) (Fig 3). Native NP cells were visualised
392 embedded within a disorganised fibrous collagen matrix, throughout the 6 week
393 culture duration in media injected control explants (Fig 3). Where MSCs were
394 injected alone clustered cells could be seen following 48 hours which appeared to
395 have migrated following 6 weeks in culture (Fig 3). Injected acellular L-pNIPAM-co-
396 DMAc hydrogel displayed a comparatively uniform interconnecting porous network
397 encapsulated by the surrounding NP tissue following 6 weeks in culture. Where
398 MSCs were incorporated within the L-pNIPAM-co-DMAc hydrogel prior to injection,
399 cells could be seen within the centre of the hydrogel region (Fig 3); a distinction in

400 the microscale morphology of the interior hydrogel was still evident in comparison to
401 the native surrounding NP tissue following 6 weeks, however integration could be
402 seen at the hydrogel/NP tissue interface with connecting NP tissue fibres penetrating
403 within the outside edges of the hydrogel region (Fig 3).

404

405 **3.3.2. Dynamic mechanical analysis**

406 MSC injected and hydrogel injected NP tissue explants displayed similar mechanical
407 properties to media injected control explants with no significant difference in the
408 elastic modulus (G'), loss modulus (G'') or tan delta ($\tan\delta$) for any of the experimental
409 groups following 6 weeks in culture (Fig 4a,b,c).

410

411 **3.3.3. Hydration assessment**

412 The injection of MSCs alone did not significantly alter the hydration degree of NP
413 tissue explants following 48 hours, 4 and 6 weeks in culture, although a significant
414 increase ($P=0.0011$) in the hydration of hMSC injected alone NP tissue explants was
415 observed in comparison to media injected control explants following 2 weeks in
416 culture (Fig 4d). Where acellular L-pNIPAM-co-DMAc hydrogel was injected a
417 significant increase ($P=0.0064$) in the hydration degree was observed in comparison
418 to media injected control explants following 6 weeks in culture (Fig 4d). Where
419 hMSCs were incorporated into L-pNIPAM-co-DMAc hydrogel prior to injection a
420 significant increase in the hydration degree was observed in comparison to media
421 injected control explants following 2 ($P=0.0015$) and 6 ($P=0.0095$) weeks in culture in
422 comparison to control explants (Fig 4d).

423

424 **3.4. Histological evaluation of matrix components**

425 Reduced matrix staining for both proteoglycans, and collagen was observed
426 throughout the 6 week culture period within media injected control NP explants (Fig
427 5). Where MSCs were injected alone, blue staining for proteoglycans was present
428 within and surrounding the cell clusters, although the staining intensity was less than
429 that of the native surrounding NP tissue (Fig 5). Areas of blue staining for collagen
430 were also observed within cell clusters where MSCs had been injected alone,
431 although the majority of cells present within the clusters were negative (red) for
432 collagen staining (Fig 5). Positive blue matrix staining for both proteoglycans and
433 collagen was observed within the acellular L-pNIPAM-co-DMAc hydrogel which had
434 been injected into NP explants, particularly following 4 and 6 weeks (Fig 5). A distinct
435 native NP tissue border, with high intensity collagen staining, was observed
436 surrounding the acellular L-pNIPAM-co-DMAc hydrogel following 48 hours, 2 and 4
437 weeks in culture (Fig 5). Blue positive proteoglycan and collagen producing cells
438 were observed within the L-pNIPAM-co-DMAc hydrogel where hMSCs had been
439 incorporated prior to injection throughout the 6 week culture duration (Fig 3). Native
440 NP tissue directly adjacent to L-pNIPAM-co-DMAc hydrogel with incorporated
441 hMSCs, demonstrated strong intensity staining for both proteoglycans and collagen
442 (Fig 5). A distinct NP tissue border, with high intensity collagen staining, was present
443 surrounding the L-pNIPAM-co-DMAc hydrogel, where MSCs were incorporated,
444 following 48 hours and 2 weeks in culture, however this was subsequently absent
445 with integration of the surrounding NP tissue within the hydrogel observed following
446 4 and 6 weeks in culture (Fig 5).

447

448 **3.5. Immunohistochemical evaluation of cell phenotype**

449 Immunohistochemistry was used to assess the expression and localisation of the NP
450 matrix markers collagen type II, aggrecan and chondroitin sulphate within MSCs,
451 both in monolayer and following injection into NP tissue explants, as well as within
452 native NP cells within the explant tissue (Fig 6). Collagen type II, aggrecan and
453 chondroitin sulphate was expressed by all native NP cells found with control,
454 acellular L-pNIPAM-co-DMAC injected, hMSC only injected and hMSC incorporated
455 within L-pNIPAM-co-DMAC injected NP tissue explants, throughout the 6 week
456 culture duration (Fig 6). Immunopositive cells for collagen type II, aggrecan and
457 chondroitin sulphate were identified surrounding the acellular L-pNIPAM-co-DMAC
458 hydrogel following 48 hours, 2 and 4 weeks in culture, with some immunopositive
459 native NP cells for collagen type II, aggrecan and chondroitin sulphate observed
460 within the acellular L-pNIPAM-co-DMAC hydrogel following 6 weeks in culture (Fig 6).
461 Monolayer MSCs extracted from culture prior to injection into NP tissue explants
462 showed no immunopositivity for aggrecan, with low levels of immunopositivity for
463 chondroitin sulphate (average 52% range 50-55%) and collagen type II (average
464 17% range 14-20%) (Suppl Fig 1). Immunopositive cellular and matrix staining for
465 collagen type II, aggrecan and chondroitin sulphate was observed within the vicinity
466 of the cell clusters where hMSCs had been injected alone, from 48 hours to 6 weeks
467 in culture (Fig 6). Intense matrix staining and positive cellular staining for collagen
468 type II, aggrecan and chondroitin sulphate was observed within the L-pNIPAM-co-
469 DMAc hydrogel where MSCs had been incorporated prior to injection (Fig 6). NP
470 tissue with high intensity matrix staining for collagen type II and chondroitin sulphate
471 was present integrating within the L-pNIPAM-co-DMAC hydrogel where MSCs had

472 been incorporated prior to injection, following 6 weeks in culture (Fig 6). All IgG
473 control sections for collagen type II, aggrecan and chondroitin sulphate showed no
474 immunopositivity (suppl Fig 1).

475 **3.6. Mechanical Characterisation of L-pNIPAM-co-DMAc hydrogel injected** 476 **Whole Bovine IVD.**

477 Whole bovine IVDs subjected to loading simulating a short period of walking
478 demonstrated a different mechanical response depending on the treatment regime of
479 the disc. When differences in disc size were accounted for and outliers excluded
480 healthy discs had 1.88 times higher stiffness (24.3 to 12.9 MPa) (Fig7A), 80% lower
481 strain (0.54% to 2.7%) (Fig 7B) and 2.8 times greater energy dissipation (2.12 to
482 0.76 J/cycle) (Fig 7C) compared to discs that had undergone collagenase digestion,
483 with $P < 0.001$ in each case when outliers were accounted for (Fig 7). Discs subjected
484 to collagenase digestion could be seen in 6 out of 10 discs to display clear digestion
485 of the IVD (Fig 7) with alteration to mechanical properties, however 4 discs which
486 were injected with collagenase failed to show evidence of digestion (no voids visible)
487 and thus were removed from the statistical analysis and showed as red outliers (Fig
488 7). Discs subjected to the same collagenase treatment process following observation
489 of clear evidence of digestion morphologically were subsequently injected with L-
490 pNIPAM-co-DMAc hydrogel, these discs demonstrated a complete recovery of disc
491 stiffness, displaying only non-significant ($P > 0.05$) differences from healthy disc
492 stiffness (24.3 to 26.4 MPa) (Fig 7A) and strain values (0.54% to 0.5%) (Fig 7B).
493 However there was no recovery of the discs ability to dissipate energy (Fig7C), with
494 hydrogel injected discs dissipating less energy than any other test group (0.40
495 J/cycle), significantly ($P < 0.001$) lower than even collagenase digested discs (Fig 7c).
496 Discs injected with hydrogel containing green dye could be clearly identified

497 macroscopically (Fig 7D,E) and microscopically were shown to fill the fissures
498 formed via collagenase digestion (Fig 7F,G).

499

500 **4. Discussion**

501 **4.1. Evaluation of biological performance to act as a cell delivery vehicle.**

502 The clinical translation of hydrogels specifically developed for the delivery of
503 regenerative cells to the IVD is dependent on several requirements: that they can be
504 administered by a minimally invasive procedure that delivers the required cell
505 population without detrimental effects to both the implanted cells and surrounding
506 tissues during delivery. That they are biocompatible and thus support the viability of
507 delivered cells and native cell populations. **The hydrogel should** facilitate cellular
508 migration to aid in the integration with surrounding tissue to prevent biomaterial
509 extrusion. Finally, **the hydrogel should** support and promote the differentiation of
510 delivered MSCs into biologically functional NP like cells in order to repair and
511 regenerate a matrix akin to native NP tissue.

512 **4.1.2. Minimally Invasive delivery of L-pNIPAM-co-DMAC hydrogel**

513 In our previous *in vitro* studies we have demonstrated that hMSC could be
514 incorporated into liquid L-pNIPAM-co-DMAC hydrogel, injected through a narrow 26-
515 gauge needle before solidification at 37°C with no detriment to cell viability [23,33].
516 Here, acellular L-pNIPAM-co DMAC hydrogel was injected through the AF into
517 collagenase digested bovine IVD via 26-gauge needle injection. The hydrogel was
518 maintained within the disc during mechanical loading and shown histologically to
519 infiltrate micro and macro fissures akin to those which could occur during IVD

520 degeneration. The selected needle diameter is an important design consideration
521 which is often dictated by the viscosity of the biomaterial being injected [34]. It is
522 generally accepted that the needle diameter should be as narrow as possible to
523 avoid structural and mechanical damage to surrounding tissues during injection.
524 However decreasing the needle diameter for viscous biomaterials increases injection
525 pressures which can increase shear forces on cells reducing cell viability [34,35].
526 The low viscosity (0.97MPas at 54°C) [23] of the L-pNIPAM-co-DMAC liquid hydrogel,
527 enabled its injection with incorporated hMSCs into NP tissue explants with no loss in
528 viability. The minimally invasive route demonstrated here offers significant advantage
529 over previously developed hydrogels implanted into IVD tissue where nucleotomy
530 was required to create a void for the biomaterial to occupy [13-16,36], or large
531 diameter surgical implanting tools were used [37]. Moreover, the synthetic route
532 utilised, which exploits the thermal phase transition of a fully reacted polymer in the
533 liquid state, offers rapid solidification at 37°C. This maintains the L-pNIPAM-co-
534 DMAC hydrogel and incorporated hMSCs within the injection site, whilst avoiding the
535 need for additional implantation devices to initiate *in situ* polymerisation such as
536 those proposed for photopolymerised hydrogels [15,25,34].

537 **4.1.3. Biocompatibility of L-pNIPAM-co-DMAc hydrogel.**

538 The biocompatibility of delivered hMSCs is essential if the repopulation of cells for
539 the long term recovery and regeneration of a functional NP matrix is to be a viable
540 therapeutic option in the treatment of IVD degeneration. In agreement with previous
541 studies [17,20,38-42], excellent viability across all time points and all treatment
542 regimes were observed. However, despite mimicking the hypoxic disc
543 microenvironment, it should be noted that the culture conditions used in this study do
544 not completely reflect that of the native IVD in terms of a mechanically loaded

545 environment [43] and low nutrient supply [44]. Moreover, the degenerate IVD is an
546 extremely hostile biological environment with increased production of matrix
547 degrading enzymes [6] and pro-inflammatory cytokines [8,9] which may affect
548 viability *in vivo*. Future investigations to ascertain the survival of delivered MSCs
549 within such conditions is paramount to the clinical translation of this kind of therapy.
550 However the survival of MSCs incorporated within the L-pNIPAM-co-DMAc hydrogel,
551 is extremely promising since the use of the hydrogel as a delivery system also
552 provides the opportunity to simultaneously deliver antagonists of catabolic mediators
553 if required.

554 **4.1.4. Integration of L-pNIPAM-co-DMAc hydrogel.**

555 The infiltration of native NP cells, demonstrated in both acellular and hMSC
556 incorporated within the L-pNIPAM-co-DMAc hydrogel injected into NP explants, is
557 particularly important to aid in scaffold integration with the surrounding NP tissue, to
558 restore optimum mechanical function and prevent issues such as biomaterial
559 extrusion [14,45]. Integration of L-pNIPAM-co-DMAC hydrogel with surrounding NP
560 tissue was particularly evident in this study, with deposited matrix penetrating within
561 the hydrogel/tissue interface shown histologically and using SEM. Despite initial
562 fibrous encapsulation observed surrounding the hydrogel following 4 weeks in
563 acellular L-pNIPAM-co-DMAc hydrogel injected NP tissue explants, it is
564 hypothesised that dynamic culture under mechanical load would promote and
565 accelerate the hydrogel tissue integration, as dynamic compressive mechanical
566 loading has been shown to promote NP matrix biosynthesis [46], increase NP cell
567 metabolism [46,47] and promote proliferation and differentiation of MSCs into NP-like
568 cells [48,49].

569 4.1.5. Differentiation of MSCs following Injection into NP tissue explants

570 In agreement with previous studies [17], where MSCs were injected alone into NP
571 tissue explants, the cells were maintained within clusters at the vicinity of the
572 injection site for up to 4 weeks, although migration was evident following 6 weeks in
573 culture. Migration and differentiation of these cells into NP like cells is essential for
574 efficacious NP matrix repair. Here MSCs injected alone were shown to produce NP
575 matrix components collagen type II, aggrecan and chondroitin sulphate. The
576 deposition of this matrix was localised immunohistochemically to the clusters of
577 MSCs visualised, indicating that the MSCs themselves were responsible for the
578 matrix synthesis; this is in agreement with previous studies [17,20,39]. Despite this, a
579 major concern for the clinical translation of stem cell therapy for the treatment of IVD
580 degeneration is a lack of control over the differentiation capacity of these cells
581 following injection into nucleus pulposus tissue [27]. In addition, the location of these
582 cells following injection is crucial since MSC leakage followed by undesirable bone
583 formation has been reported previously as a potential side effect of this therapeutic
584 strategy [26]. The incorporation of MSCs within the L-pNIPAM-co-DMAc hydrogel
585 during the liquid phase for delivery, is advantageous as it ensures the
586 interconnecting porous hydrogel network is able to assemble around the cells. This
587 ensures that in the initial weeks following injection that the cells are maintained
588 within the injection site and that the microenvironment of the hydrogel itself will be
589 the first structural influence on the differentiation capacity of the MSCs. This gives a
590 greater potential control over the differentiation and location of the delivered
591 regenerative cells. We have previously demonstrated *in vitro* that MSCs incorporated
592 into L-pNIPAM-co-DMAc hydrogel and cultured under hypoxic conditions induces
593 differentiation of MSCs into NP like cells without the need for additional

594 chondrogenic inducing medium or growth factors [23]. Here, we have shown that
595 cells incorporated into L-pNIPAM-co-DMAc hydrogel and injected into NP explants
596 were shown to produce NP matrix components: collagen type II; aggrecan and
597 chondroitin sulphate. We are unable to ascertain whether the matrix deposition itself
598 is from the delivered MSCs or native NP cells, it is most likely to be a combination of
599 both since positive cells and deposited matrix which compositionally reflects native
600 NP tissue, was observed both within the hydrogel itself and surrounding the
601 hydrogel/tissue interface. Thus the translation of our previous *in vitro* results within
602 an *ex vivo* NP tissue explant model demonstrated here, offers significant promise for
603 the efficacy of this therapeutic strategy in the delivery of MSCs for the repair and
604 regeneration of the NP as a future treatment of IVD degeneration. A short coming of
605 the current study is that commercial MSCs were utilised, thus future investigations
606 should be conducted to assess the differentiation of MSCs from a large cohort of
607 human patients to determine patient variability and age of patients which can be
608 utilised for such a therapy.

609

610 **4.2. Evaluation of mechanical properties to act as a support scaffold:**

611 The NP is regarded as a viscoelastic material, exhibiting both fluid and solid like
612 behaviours [50]. It has been well documented that the viscoelasticity of NP tissue
613 changes with ageing and degeneration, exhibiting a more 'solid-like' than 'fluid-like'
614 behavior, reflected by a decreased $\tan\delta$ [51]. One of the hallmarks of IVD
615 degeneration is a reduction in the overall proteoglycan content [7] resulting in
616 reduced tissue hydration [4,52,53]. The injection of a hydrogel biomaterial, defined
617 as a 3D hydrated crosslinked polymeric network, has therefore been hypothesised
618 as an appealing strategy to restore NP tissue hydration and thus potentially regain

619 some of the viscoelastic NP material properties [54,55]. The significant increase in
620 hydration degree of NP tissue explants following 6 weeks where acellular and hMSC
621 incorporated L-pNIPAM-co-DMAc hydrogel was injected, provides promising
622 evidence to suggest that the L-pNIPAM-co-DMAc hydrogel material itself is
623 compositionally advantageous to the overall NP tissue hydration. The benefits of
624 which would be even more apparent within degenerate tissue. Despite the increased
625 hydration degree no significant difference in the G' , G'' or the $\tan\delta$ were observed
626 regardless of whether hMSCs or L-pNIPAM-co-DMAc hydrogel were injected. This is
627 likely due to the fact that the NP tissue explant controls used in this this study were
628 not experimentally manipulated to be degenerate; thus the fact that there is no
629 statistical difference in the viscoelastic parameters assessed between the different
630 experimental groups, indicates that both hMSC and L-pNIPAM-co-DMAc hydrogel
631 injected NP tissue explants display similar biomechanical properties to native NP
632 tissue. A limitation that should be considered however is that in this study the NP
633 tissue explants were statically cultured within a semi-constrained Perspex ring
634 culture system, therefore the biomechanical properties of the explant NP tissue is
635 likely to be different from native NP tissue found within the body[49,56].

636 Loading of whole bovine collagenase digested IVDs injected with L-pNIPAM-co-
637 DMAc hydrogel, demonstrated a significant recovery of structural properties,
638 completely regaining pre-digested levels of disc stiffness. The full recovery of disc
639 mechanical response following hydrogel injection, shown in this study, is a highly
640 promising result, providing supporting evidence that the hydrogel could offer a
641 significant and near immediate structural benefit to degenerate IVDs.

642 Unfortunately modelling degeneration in the disc is problematic, and whilst
643 collagenase digestion targets the collagen matrix within the disc the proteoglycan

644 components would still be non-digested. However as the collagen matrix forms the
645 interconnecting network which holds the proteoglycan proteins in place, it was
646 observed that the majority of collagenase digested discs generated large voids both
647 macro and microscopically mimicking at least in part morphological features of
648 degeneration. A limitation of the current study was that digestion of the discs using
649 collagenase did not always induce digestion (4 out of 10 discs) and these samples
650 failed to show any mechanical response. Therefore when performing the hydrogel
651 injection following collagenase digestion, discs were carefully examined to ensure
652 voids were visible prior to hydrogel insertion. Following loading, histological analysis
653 demonstrated collagenase digested voids were filled with hydrogel, in all discs
654 investigated.

655 The reduced stiffness of collagenase digested discs and subsequent recovery
656 following the introduction of the L-pNIPAM-co-DMAc hydrogel system may initially
657 seem counter intuitive. Damage and degeneration of the NP expected to reduce
658 'fluid-like' behaviour of the disc in favour of stiffer, 'solid-like' behaviour [4,51,53]. The
659 collagenase digestion process resulted in the creation of voids or fissures within the
660 NP, as evidenced by macro and microscopic imaging of tissues, but minimal
661 structural damage to the AF. The result, *in vitro*, is a disc which maintains its original
662 height yet contains easily compressible voids, resulting in reduced bulk stiffness.
663 More significant breakdown of disc tissue or full or partial excision of the disc nucleus,
664 particularly when combined with long term, continuous pressure on the IVD *in vitro*
665 [57] is liable to compress voids, reducing disc height and resulting in a thin disc of
666 mostly AF tissue, which is stiffer than that of the NP [58]. This non-linear variation in
667 disc stiffness with degeneration grade has previously been observed in torsion,
668 flexion and bending [59,60]. When comparing stiffness of moderately degenerate

669 discs compressed *in vitro* to degenerate discs *in vivo* it may be more accurate to look
670 at the results in terms of potential to lose disc height, an important biomarker of disc
671 degeneration [23-27].

672 Interestingly, significantly lower energy dissipation was observed in groups injected
673 with the needle: sham injected; collagenase digested; and collagenase digestion
674 followed by hydrogel injection, compared with healthy whole bovine IVDs. **In contrast**
675 **Michalek & Iatridis, (2012) observed no apparent difference in pressurization testing**
676 **following needle puncture of bovine motion segments [61]. Interestingly however the**
677 **study by Michalek & Iatridis (2012) induced a 10mm deep defect within the AF which**
678 **would not have lead to puncture of the NP unlike the current study where needle**
679 **puncture was performed to the centre of the disc and thus into the NP region [61].**
680 **Furthermore the loading rate investigated by Michalek & Iatridis, (2012) was**
681 **considerably lower (0.1Hz) compared to the 2Hz used in the present study [61]. In**
682 **the present study these dynamic parameters were selected to mimic that of walking**
683 **in the human spine to enable a functional assessment of the treatment methods. The**
684 **loss in energy disipation seen in the current study following needle puncture through**
685 **the AF and into the NP together with the results of Carragee *et al.*, 2009, suggests**
686 **that the needle puncture itself in healthy IVDs may be the key initiator of discs losing**
687 **tissue functionality, likely due to a loss in the pressure of the IVD [62]. **However, as****
688 **the degenerate **human** disc **will** already have a reduced energy dissipation capacity**
689 **due to matrix degradation and presence of fissures [51,63,64]. The delivery of the L-**
690 **pNIPAM-co-DMAc hydrogel system via needle injection, is not likely to initiate any**
691 **further loss in energy dissipation but will provide an immediate mechanical and**
692 **clinical benefit in terms of IVD stabilisation, to what is likely to be, at the time of**
693 **patient treatment, a mechanically dysfunctional tissue.**

694 The injection of acellular L-pNIPAM-co-DMAc hydrogel injection did not recover
695 energy dissipation to pre-digested levels; however the energy dissipation
696 functionality is reliant on the unique pressurised environment of the intact IVD [65],
697 therefore it is unlikely that the implantation of a biomaterial alone would provide this
698 biomechanical function immediately. It is hypothesised that over time as the L-
699 pNIPAM-co-DMAc hydrogel integrates with surrounding NP tissue, whilst the
700 delivered MSCs simultaneously repair and regenerate a biologically functioning NP
701 matrix to fill in any potential fissures, that the pressurised environment and energy
702 dissipation function of the IVD will be restored.

703 The mechanical benefit of hydrogel injection within IVDs has been previously
704 reported, including the restoration of the load transmission [36] and re-established
705 disc height maintained over 0.5 million loading cycles within a bovine organ culture
706 model [15], however in both cases, nucleotomy was required for hydrogel
707 implantation and the incorporation and delivery of cells was not investigated.
708 Recently Balkovec *et al.*, 2016 reported the restoration of segmental kinematics to
709 pre injury state in IVDs with disc height loss, following hydrogel injection, within an *ex*
710 *vivo* porcine cervical spine model; however again nucleotomy was required to inject
711 the hydrogel, the incorporation and delivery of cells was not investigated [13] and the
712 immediate mechanical restoration of the hydrogel was not maintained following
713 cyclic loading [14].

714 The development of a biomaterial which offers the ability to safely deliver and
715 differentiate cells, without the use of additional growth factors, as well as providing
716 mechanical stabilisation, has so far not been achieved. Here, we demonstrate that
717 the L-pNIPAM-co-DMAc hydrogel can be delivered by minimally invasive injection
718 into collagenase digested whole bovine IVDs. Where it fills micro and macro fissures,

719 is maintained within the disc during loading and provides immediate mechanical
720 stabilisation with improved disc stiffness back to non-digested levels, without the
721 need for prior removal of the NP tissue. Future investigations are required to assess
722 the fatigue properties of the hydrogel following prolonged cyclic loading. The
723 promising results displayed here suggest that the L-pNIPAM-co-DMAc hydrogel
724 could provide a treatment strategy which requires a less invasive surgical
725 intervention, in regards to removal of native tissue, which may promote better tissue
726 integration and tissue functionality. Moreover the ability to safely deliver and promote
727 NP differentiation of MSCs within the L-pNIPAM-co-DMAc hydrogel system also
728 provides the opportunity to biologically repair the disc as well as providing
729 mechanical stability.

730 **4.3. Treatment design strategy tailored for stage of degeneration.**

731 Our improved understanding of the underlying pathogenesis of IVD degeneration
732 over recent years and the consequential morphological changes that occur should
733 be conveyed in our treatment design strategies [21,22]. It is possible that clinicians
734 could be presented with a variety of treatment options, both cellular and acellular, in
735 order to deliver the most efficacious, safe and cost effective treatment for the stage
736 of degeneration [21]. Of course clinical translation is reliant on the detection and
737 diagnosis of patients with early to mid-stages of IVD degeneration; however recent
738 advances in quantitative MRI imaging give future promise to this possibility [66].
739 Future investigations into the survival and differentiation of MSCs within the hostile
740 environment of a severely degenerate IVD are crucial in determining the success of
741 stem cell intervention for these patients. Additionally hydrogel containment and
742 mechanical stability within severely degenerate IVDs, where annular fissures,
743 osteophytes and endplate fractures may be clinically present, must also be

744 investigated to assess whether hydrogels can offer clinical benefit for late stage IVD
745 degeneration. The results of these investigations, in combination with the design of
746 suitable biomaterials and diagnostic techniques will determine the future possibility of
747 personalized therapies for IVD degeneration.

748 **5. Conclusion**

749 Here, we have investigated the efficacy of a range of treatment options: hMSCS
750 injected alone, acellular L-pNIPAM-co-DMAc and hMSCs incorporated within the L-
751 pNIPAM-co-DMAc hydrogel, injected into bovine NP tissue explants. Demonstrating
752 that hMSCs injected alone or incorporated within the L-pNIPAM-co-DMAc hydrogel
753 are able to differentiate and produce NP matrix components, thus providing
754 compelling evidence in support of cell delivery for NP matrix repair. Additionally we
755 have demonstrated immediate mechanical stabilisation with the injection of acellular
756 L-pNIPAM-co-DMAc hydrogel into whole bovine IVDs, demonstrating a potential
757 clinical benefit even in the absence of cells. The delivery of the L-pNIPAM-co-DMAc
758 hydrogel system via minimally invasive 26 gauge needle injection and its ability to fill
759 micro fissures, without the removal of the existing NP tissue, provides the
760 opportunity to target symptomatic patients in early to mid-stages of degeneration.
761 The use of a combined cellular and mechanical repair approach is particularly
762 promising since it is hypothesised that the L-pNIPAM-co-DMAc hydrogel, could
763 restore disc height, thus providing immediate pain relief, whilst delivery of MSCs
764 provides gradual regeneration.

765 **5. Author contributions**

766 AAT performed the majority of the laboratory work (except for the mechanical testing
767 of whole bovine IVDs), data analysis and statistical analysis, contributed to study

768 design and drafted the manuscript. GD performed the laboratory work and data
769 analysis for the mechanical testing of whole bovine IVDs and helped draft the
770 manuscript. LV assisted in the experimental set up of bovine NP tissue explants and
771 critically revised the manuscript. NS and GC participated in the design and
772 coordination of the mechanical testing experiments on whole bovine IVDs, aided in
773 the analysis of data and critically revised the manuscript. CS and CLLM conceived
774 the study, participated in its design and coordination, aided in the analysis of data,
775 secured funding and critically revised the manuscript. All authors read and approved
776 the final manuscript.

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783 **7. Disclosure**

784 No conflicts of interest to declare.

785 **Figure Legends:**

786 **Figure 1:** Photomicrographs of haematoxylin & eosin stain, Hoechst staining and
787 green fluorescent protein CFSE positive cells (shown inset for hMSC and hMSC +
788 Hy NP tissue explants) in the injection sites of bovine nucleus pulposus tissue
789 following 48 hours, 2, 4 and 6 weeks in culture. Magnified images of native NP cells,

790 stained with Hoechst, surrounding acellular hydrogel, shown inset to aid visualisation.
791 White arrows on acellular Hy images indicate cells, black arrows on H&E images
792 indicate hydrogel/tissue interface, red arrows on H&E images for hMSC + Hy
793 indicate cells within hydrogel. Media injected control tissue (Control), CFSE positive
794 hMSC injected alone (Injected hMSC), L-pNIPAM-co-DMAc hydrogel injected
795 without cells (Acellular Hy) and hMSC incorporated into L-pNIPAM-co-DMAc
796 hydrogel injected (hMSC+ Hy). *Scale bar = 50 μ m or 100 μ m.*

797 **Figure 2:** Immunohistochemical detection of caspase 3 to assess cell viability after
798 48 hours, 2, 4 and 6 weeks in culture. Percentage immunopositivity was calculated
799 (A). Photomicrographs representative of caspase 3 immunopositivity in tissue
800 explants at 48 hours and 6 weeks post injection (B). Back arrows indicate positively
801 stained cells and white arrows indicate negatively stained cells. *Scale bar = 100 μ m.*

802 **Figure 3:** Scanning Electron Microscopy (SEM) of media injected control (Control),
803 hMSC injected alone (hMSC), Acellular L-pNIPAM-co-DMAc hydrogel injected
804 (Acellular Hy) and hMSC incorporated within L-pNIPAM-co-DMAc hydrogel injected
805 (hMSC + Hy) NP tissue explants following 48 hours and 6 weeks in culture. Enlarged
806 images of native NP cells (control), hMSCs (hMSCs injected alone) and cells within
807 hydrogel region (Acell 6 weeks, hMSC + Hy 48 hours and 6 weeks) shown inset for
808 visualisation. White arrows indicate native NP cells within Control NP explants and
809 presence of hMSCs within hMSC injected alone NP explants. Blue arrows indicate
810 NP tissue/hydrogel interface, demonstrating integration of hydrogel with surrounding
811 NP tissue. *Scale bar 50 μ m.*

812 **Figure 4:** Mechanical Analysis of bovine explant NP tissue using dynamic
813 mechanical analysis (A,B,C). (A) Elastic modulus (G'), (B) loss modulus (G'') and (C)

814 Tan Delta ($\tan\delta$) at 2.5Hz after 6 weeks in culture. (D) Calculated hydration degree
815 of bovine explant tissue throughout 6 weeks in culture. Media injected control (con),
816 hMSC injected alone (hMSC), acellular L-pNIPAM-co-DMAc injected (Acell Hy) and
817 hMSC incorporated into L-pNIPAM-co-DMAc injected (hMSC + Hy). (*) Indicates
818 statistical significance compared to controls at each timepoint (D) ($*p = \leq 0.05$).

819 **Figure 5:** Histological evaluation of bovine NP tissue explants using Alcian blue (AB)
820 for proteoglycan deposition and Masson trichrome (MT) for collagen deposition after
821 48 hours, 2, 4 and 6 weeks in culture. Media injected control tissue (control), hMSCs
822 injected alone (hMSC), Acellular L-pNIPAM-co-DMAc injected (Acellular Hy) and
823 hMSCs incorporated within L-pNIPAM-co-DMAc hydrogel injected (hMSC + Hy).
824 Black arrows indicate positively stained cells, red arrows indicate negatively stained
825 cells and orange arrows indicate hydrogel/NP tissue interface. Magnified images of
826 representative cells shown inset for visualisation. *Scale bar = 100 μ m.*

827 **Figure 6:** Immunohistochemical detection of NP matrix markers collagen type II,
828 aggrecan and chondroitin sulphate in bovine NP tissue explants. Representative
829 photomicrographs after 48 hours, and 6 weeks in culture. Black arrows demonstrate
830 positively stained cells and green arrows demonstrate negatively stained cells.
831 Enlarged immunopositive cells shown inset for visualisation. *Scale bar = 100 μ m.*

832 **Figure 7:** Mechanical analysis of whole bovine IVDs that were healthy, stabbed with
833 21G needle (sham), collagenase digested or collagenase digested followed by
834 hydrogel injection (hydrogel). Measured parameters include: (A) Young's Modulus,
835 (B) % Strain and (C) Energy Dissipation. Four discs (red) from the digested group
836 demonstrated young's modulus and strain behaviour similar to sham injected
837 suggesting collagenase injection had been insufficient and this was supported by the

838 lack of morphological evidence of collagenase digestion in these discs, thus were
839 marked as outliers but Energy Dissipation was in line with other needle punctured
840 discs. All replicates have been shown with outliers marked in red to demonstrate
841 clearly the spread of replicates. Median values indicated do not include outliers
842 within digested group. (*) Indicates significant differences between experimental
843 groups ($p \leq 0.05$). (D) Representative macroscopic image of whole bovine IVD
844 following collagenase digestion (average IVD diameter 30mm). (E) Representative
845 macroscopic image following collagenase digestion and hydrogel injection, with a
846 green food dye incorporated within the hydrogel to aid visualisation. (F, G)
847 Microscopic images stained with haematoxylin and eosin of whole IVD following
848 collagenase digestion, hydrogel injection and mechanical loading. *Scale bar 1000 μ m,*
849 *200 μ m.*

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851 **References**

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