



# CHITOSAN-BASED HYDROGELS SUPPLEMENTED WITH GELATINE AND LINK N ENHANCE EXTRACELLULAR MATRIX DEPOSITION BY ENCAPSULATED CELLS IN A DEGENERATIVE INTERVERTEBRAL DISC ENVIRONMENT

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## Abstract

Injectable therapies for intervertebral disc (IVD) repair are gaining much interest. Recently, a chitosan (CH)-based injectable scaffold has been developed that has similar mechanical properties to human nucleus pulposus (NP) and provides a suitable environment for encapsulated NP cell survival and proteoglycan production. The hypothesis of the study was that the biological response of the encapsulated cells can be further increased by adding gelatine and Link N (LN, a naturally occurring peptide present in cartilage and IVD extracellular matrix), known to increase cell adhesion and proteoglycan production, respectively. The effect of gelatine on the mechanical properties of a CH hydrogel was evaluated through rheological and compressive mechanical tests. Production of proteoglycan [assessed as glycosaminoglycan (GAG)] by encapsulated NP cells was determined in the presence or absence of gelatine in normal or degenerative medium supplemented with LN. Normal and degenerative media replicate the healthy and degenerative disc environment, respectively. Gelatine slightly reduced the gelation rate of CH hydrogel but improved its final mechanical properties in compression. LN had a minimal effect in normal medium but induced significantly more GAG production in degenerative medium ( $p < 0.001$ , 4.7-fold superior to the control), reaching similar results to transforming growth factor (TGF)- $\beta$  (used as a positive control). GAG production was further increased in CH-gelatine hydrogels, confirming an additive effect of LN and gelatine in a degenerative environment. The results supported the concept that CH-gelatine hydrogels supplemented with LN can help restore the function of the NP during the early stages of IVD degeneration.

**Keywords:** Chitosan, intervertebral disc, Link N, gelatine, injectable hydrogel, nucleus pulposus.

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| List of Abbreviations |   |               |                                  |
|-----------------------|---|---------------|----------------------------------|
| BGP                   | $\beta$ -glycerophosphate disodium salt hydrate | IL            | interleukin                      |
| BMP                   | bone morphogenetic protein                      | IPN           | interpenetrating polymer network |
| CH                    | chitosan  | IVD           | intervertebral disc              |
| DMEM                  | Dulbecco's modified Eagle's medium              | IVDD          | intervertebral disc degeneration |
| DMMB                  | dimethyl methylene blue                         | LN            | Link N                           |
| ECM                   | extracellular matrix                            | LVE           | linear viscoelastic range        |
| FAM                   | carboxyfluorescein                              | NP            | nucleus pulposus                 |
| FBS                   | foetal bovine serum                             | OP-1          | osteogenic protein 1             |
| GDF-5                 | growth differentiation factor 5                 | PBS           | phosphate-buffered saline        |
| IGF                   | insulin-like growth factor                      | SD            | standard deviation               |
|                       |   | SHC           | sodium hydrogen carbonate        |
|                       |   | TGF- $\beta$  | transforming growth factor beta  |
|                       |   | TNF- $\alpha$ | tumour necrosis factor alpha     |

## Introduction

IVDD is a leading cause of chronic back pain and loss of function. It is a multifactorial disease, characterised by the loss of ECM, specifically proteoglycan and collagen, tissue dehydration, fissure development, loss of disc height, inflammation, endplate sclerosis, cell death and hyperinnervation of nociceptive nerve fibres (Freemont, 2009). There are no therapeutics for IVDD and the current first line treatment modalities are focused on pain management, but none target the underlying causes. Inadequately controlled pain is the main reason for invasive procedures such as discectomy, laminectomy, disc replacement and spinal fusion (Wu *et al.*, 2020). Over 1.6 million instrumented surgical procedures are performed annually in the United States (Web ref. 1). Yet, despite the frequency of the most common procedure, spinal fusion, clinical success varies from 50 to 70 %. Pain relief is often short-lived and the altered biomechanics can lead to adjacent disc degeneration (Lee *et al.*, 2015; Nguyen *et al.*, 2011). Consequently, there is an unmet need for better and efficacious approaches that deal directly with the pathological state of the degenerated IVD.

One of the most important changes caused by IVDD is the loss of proteoglycans residing in the NP. These proteoglycans are hydrophilic glycoproteins responsible for attracting and retaining water molecules inside the disc (Shapiro and Risbud, 2013). Their high-water concentration confers important compressive capacity to the NP tissue, which is able to absorb and adequately distribute compressive loads to the spine (Gilchrist *et al.*, 2011; O'Halloran and Pandit, 2007). Due to age and not well-known causes, NP proteoglycan content decreases gradually (Raj, 2008), leading to a reduction in the osmotic pressure of the discal matrix and an inadequate load redistribution leading to IVDD. Since it is difficult for the native NP to achieve self-renewal, any treatment which can enhance proteoglycan content in the NP may help to slow or stop the degeneration process and eventually regenerate the biomechanical properties of the disc (Iatridis *et al.*, 2013).

Injectable cell therapy targeting the central gelatinous NP is a promising approach for biological repair. Various studies were intended to design injectable scaffolds containing cells and eventually a bioactive agent to optimise their function (Frith *et al.*, 2013; Smith *et al.*, 2014; Thorpe *et al.*, 2016). An ideal injectable scaffold should provide a suitable environment for encapsulated cells to survive and produce ECM components while exhibiting similar mechanical properties to native tissue. Assaad *et al.* (2015) have recently developed a scaffold that fulfils these requirements. This scaffold is an injectable thermoresponsive CH-based hydrogel that rapidly gels at body temperature. Thanks to the use of a combination of SHC and BGP, it presents similar mechanical properties to human NP and provides a

suitable environment for the survival of encapsulated NP cells. However, previous results have shown a suboptimal synthesis of proteoglycans (Alinejad *et al.*, 2019).

A strategy to increase proteoglycans synthesis by encapsulated cells is the addition of cell adhesive components in the hydrogel. Gelatine, obtained by denaturation of collagen (Gentile *et al.*, 2016), is well known to increase cell proliferation (Xia *et al.*, 2004) and adhesion through binding motifs recognised by cell integrin receptors (Huang *et al.*, 2005; Zheng *et al.*, 2018). Gilchrist *et al.* (2007) have shown that human disc cells attach to the collagen present in the ECM through alpha integrin receptors. The authors have also demonstrated the impact of mechanical properties and ECM ligands on NP cell activity. Indeed, they hypothesised that the presence of appropriate ECM ligands such as collagen and laminin in a suitable mechanical environment can lead to a higher proteoglycan production (Gilchrist *et al.*, 2011). Based on those studies and the fact that gelatine is non-immunogenic (Jaipan *et al.*, 2017), the study hypothesis was that adding gelatine in the CH hydrogel would improve cell attachment to the scaffold, increase cell survival and lead to enhanced proteoglycan synthesis.

In addition to cell adhesive components, growth factors such as TGF- $\beta$ , GDF-5, BMP-2, IGF, OP-1 (or BMP-7) have been shown to increase the production of proteoglycans by disc cells (Alini *et al.*, 2003; Bach *et al.*, 2017; Kim *et al.*, 2003; Smith *et al.*, 2014). However, these bioactive agents may not be suitable for clinical applications, due to their risk of tumorigenicity (Brahmkhatri *et al.*, 2015) and prohibitive cost. Therefore, LN peptide was proposed as an alternative.

LN is a naturally occurring 16 amino acid peptide representing the N-terminal region of the Link protein, an endogenous glycoprotein that binds hyaluronic acid and aggrecan in both the IVD and articular cartilage and stabilises the resulting proteoglycan aggregates (Melching and Roughley, 1985). Previous studies have demonstrated that LN meets the criteria for an ideal IVDD treatment as it acts as an anabolic agent, reversing structural degradation of the IVD due to the disease, and blocks the inflammatory and pain-producing pathways (Antoniou *et al.*, 2012; Bach *et al.*, 2017; Gawri *et al.*, 2012; Gawri *et al.*, 2014; Mwale *et al.*, 2011; Noorwali *et al.*, 2018).

The objective of this study was to design an injectable therapy for IVD repair by associating the CH thermosensitive hydrogel with gelatine and LN as bioactive agents and investigate the effect of gelatine or/and LN addition on proteoglycan synthesis by encapsulated NP cells in CH hydrogels *in vitro*. First, rheological and compressive mechanical tests were conducted to assess the potential effect of gelatine on mechanical and gelation properties. Secondly, gelatine and/or LN effects on encapsulated NP cells

survival and proteoglycan synthesis were evaluated in a condition replicating a healthy or degenerative disc environment.

## Materials and Methods

### Source of CH, gelatine and LN

CH powder (Kitomer, PSN 32850, molecular weight 250 kDa, 95 % deacetylated) was supplied by Marinard Biotech (Rivière-au-Renard, QC, Canada), while gelatine powder from porcine skin was obtained from Sigma-Aldrich (gelatine type A, G1890). LN (DHLSDNYTLDDHRAIH) was synthesised with a purity > 98 % by CanPeptide (Pointe Claire, QC, Canada).

### Preparation of CH and CH-gelatine hydrogels

CH powder with or without gelatine was solubilised in 0.1 mol/L of HCl. Obtained CH or CH-gelatine solutions were autoclaved (121 °C for 20 min) and stored at 4 °C until use. A gelling agent solution consisting of BGP (G9422, Sigma-Aldrich) and SHC (144-55-8, EMD Millipore Corporation) was prepared using deionised water and filtered through a 0.22 µm membrane. Hydrogel solutions were prepared by mixing CH or CH-gelatine solutions with the gelling agent solution at a volume ratio of 3 : 2 using two syringes and a Luer Lock (H93813904, Baxter, Zurich, Switzerland) syringe connector as previously described (Assaad *et al.*, 2015). Tested formulations contained constant CH 2 % w/v and gelling agent concentrations (75 mmol/L SHC + 100 mmol/L BGP), with different concentrations of gelatine (0, 1, 1.5 and 2 % w/v).

### Hydrogel gelation kinetics

Two different rheological tests were performed using a Physica MCR 301 rheometer (Anton Paar, Ostfildern, Germany) equipped with coaxial cylinder geometry (CC10/T200). First, the stability at room temperature and the thermoresponsivity of the hydrogels were evaluated. Just after preparation, 1.5 mL of hydrogel solution was poured into the rheometer. Storage ( $G'$ ) and loss ( $G''$ ) moduli were monitored for 20 min at room temperature (22 °C), followed by 20 min at body temperature (37 °C). For the second test, the hydrogel solution was placed in the sample holder previously heated to 37 °C and the gelation kinetic at body temperature was assessed by monitoring the evolution of the storage and loss moduli for 1 h. Both oscillatory tests were performed in the LVE, at 1 Hz frequency and 1 % strain (for stability test) or 5 % strain (for gelation kinetic test).

### Unconfined compression

CH and CH-gelatine hydrogels solutions were prepared, poured into cylinders (14 mm internal diameter) and left to gel at 37 °C for 24 h. The hydrogel discs obtained (approximately 9 mm height) were used to measure unconfined compression

properties. Compression tests were performed on each sample using a Mach-1™ Micromechanical System (Biomomentum, Laval, Canada) equipped with a 250 N load cell. A deformation rate of 100 %/min (approximately 0.15 mm/s) was applied until 70 % deformation to obtain stress-strain curves and to assess the secant modulus. The 15 % secant modulus was calculated as the slope of a line connecting the point of zero strain to the 15 % deformation point.

### Isolation of NP cells

Bovine NP cells were isolated from fresh coccygeal IVDs obtained from 2-4 years old steers within 24 h of slaughter, as previously described (Alinejad *et al.*, 2019; Gawri *et al.*, 2012). Briefly, IVDs were dissected from the adjacent vertebral bodies and washed in PBS containing 100 µg/mL penicillin, 100 U/mL streptomycin, 50 µg/mL gentamicin and 0.25 µg/mL Fungizone (Wisent Bioproducts, Montreal, QC, Canada). The NP regions were separated from the IVDs and cut into approximately 2 mm pieces. NP cells were enzymatically isolated from the tissue pieces by sequential digestion with 0.125 % Pronase (Sigma-Aldrich) for 30 min followed by 16 h incubation with 50 U/mL collagenase I (C-9891, Sigma-Aldrich) at 37 °C. The obtained solution was filtered using a 75 µm nylon cell strainer and the supernatant was centrifuged at 1,000 ×g for 10 min. The cellular pellet was washed twice with DMEM prior to seeding.

For all experiments, NP cells were expanded until passage 3 in DMEM (1 g/L glucose, 319-010-CL, Wisent Inc., Saint-Jean-Baptiste, CQ, Canada) supplemented with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin, with culture medium changed every 3 d.

### Encapsulation of cells in CH and CH-gelatine gels

Bovine NP cells were encapsulated in the hydrogels as follows: first, 0.6 mL of CH or CH-gelatine solutions were mixed with 0.2 mL gelling agent solution with two syringes connected using a Luer Lock; then, the obtained mixture was mixed with 0.2 mL cell suspension (10<sup>6</sup> cells/mL as final cell density for cell viability or 10<sup>7</sup> cells/mL for GAG production assessment). Thus, final composition remained 2 % w/v CH, 100 mmol/L BGP and 75 mmol/L SHC with 0, 1, 1.5 or 2 % w/v gelatine, as in mechanical characterisation tests. Then, this solution was placed in a 48-wells plate (200 µL/well) and incubated for 3 min at 37 °C. Culture medium (500 µL/well) was further added in each well and the plate was maintained in the incubator at 37 °C, 5 % CO<sub>2</sub>.

Two different culture media were tested: a normal medium (PrimeGrowth™ Disc Cells Medium, 319-515-CL, Wisent Inc.) and a degenerative medium (PrimeGrowth™ Degenerative Disc Cells Medium, 319-513-CL, Wisent Inc.), supplemented with 1 ng/mL IL-1β (SRP3083, Millipore). Each medium was supplemented with 10 % FBS and 1 % penicillin/streptomycin. The normal medium was characterised



by a physiological pH and high osmolarity and was developed to ensure optimal disc cells growth and functionality during *in vitro* culture. The degenerative medium was designed to simulate the biochemical composition of a degenerative disc (lower pH and osmolarity, higher mineral content, presence of inflammatory cytokine such as IL-1 $\beta$ ).

### LN diffusion in the gel

Before testing the effect of LN on cells, LN diffusion in the CH and CH-gelatine hydrogels when added to the medium was first confirmed. For that purpose, 50  $\mu$ g of fluorescent LN (LN labelled with 5-carboxyfluorescein, CP08245F, CanPeptide Inc., Pointe Claire, QC, Canada) in 500  $\mu$ L of complete culture medium was incubated with 200  $\mu$ L of hydrogel in 48-wells plate. After 1, 3 and 24 h incubation at 37 °C, LN concentration remaining in the medium was determined using a spectrophotometer in fluorescence mode (EX485/EM520) and a calibration curve. Then, the amount of LN diffused in the gel was determined by subtracting the remaining LN in the medium from the initial LN quantity. Then, the LN concentration in the gel was compared to the concentration expected in the case of equilibrium (71  $\mu$ g/mL), calculated by dividing the quantity of LN added in each well (50  $\mu$ g) by total volume in the well [hydrogel + medium (700 mL)].

### Effect of gelatine and LN on encapsulated NP cells

To study the effect of gelatine on NP-cell viability and GAG production, NP cells encapsulated either in CH or CH-gelatine hydrogels were cultured in normal medium for 14 d. To evaluate the effect of LN and select the optimal concentration, NP cells encapsulated in CH hydrogel were cultured in normal or degenerative medium supplemented with various concentrations (0, 10, 25 or 100  $\mu$ g/mL) of human LN for 14 d. Then, the possible additive benefits of gelatine and LN were studied by encapsulating NP cells in CH or CH-1 % gelatine hydrogels and cultured in either normal or degenerative medium supplemented with 100  $\mu$ g/mL LN. 50 ng/mL TGF- $\beta$  (PHG9204, Invitrogen) was used as a positive control.

Cell viability was assessed by live/dead assay at day 1 and 14 on NP hydrogel scaffolds. Briefly, the hydrogel was washed with PBS and incubated for 45 min at 37 °C with serum-free medium supplemented with 2  $\mu$ mol/L ethidium homodimer-3 and 1  $\mu$ mol/L calcein-AM (LIVE/DEAD™ Cell Imaging Kit reagents, R37601, Life Technologies). The hydrogel was again washed with PBS and immediately observed using an inverted fluorescent microscope (Leica DM IRB). Cell viability was calculated as the ratio of live cells (green) to total cells number (green and red). The “Analyse particles function” in ImageJ (National Institute of Health) was used for the counting.

GAG production (released into the medium and retained in the construct) was analysed using the DMMB assay (Garnjanagoonchorn *et al.*, 2007).

DNA content in the gel was measured at 14 d using the PicoGreen® assay (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Thermo Fisher Scientific) following manufacturer's protocol.

### Statistical analysis

Results are presented as mean  $\pm$  SD. Statistical analysis was performed by using one-way ANOVA followed by Dunnett's multiple comparison test for studies comparing separately the effect of gelatine or LN concentrations on GAG production. Two-way ANOVA followed by Tukey's multiple comparison test was used to analyse simultaneously the effect of gelatine, LN, TGF- $\beta$  and medium type on GAG production. A  $p < 0.05$  was considered statistically significant. Each experiment was performed a minimum three times.

## Results

### Effect of gelatine on rheological and unconfined compression properties of CH hydrogels

The CH hydrogel used presents suitable mechanical properties for NP repair, *i.e.* quick gelation and compressive properties close to human NP tissue (Alinejad *et al.*, 2019). However, GAG production by encapsulated NP cells was suboptimal compared to *in vivo* levels. In the present study, gelatine, a biodegradable and biocompatible protein, was added to the hydrogel, because of its cell adhesion properties, which may increase ECM deposition by NP cells. Gelatine was also chosen because of its nonantigenic characteristics when compared to collagen (Liang *et al.*, 2004) and its physicochemical performances that can be modulated appropriately. However, adding gelatine to CH hydrogels can affect their mechanical properties, which are already suitable for the application. Therefore, the first step of the study consisted in evaluating the effect of various concentrations of gelatine (1-2 w/v %) on the scaffold gelation kinetics and compressive properties (Fig. 1).

All hydrogel formulations showed a relatively stable storage modulus ( $G'$ ) at room temperature and sudden increase in  $G'$  when the temperature was increased up to 37 °C, confirming that they were all thermosensitive (Fig. 1a). However, gelatine had a dose-dependent impact on the rheological properties. Initial  $G'$  (and viscosity, data not shown) at 22 °C increased slightly with gelatine concentration. Moreover, the increase in  $G'$  with temperature was slower for CH-gelatine hydrogels, suggesting that gelatine addition slowed down the gelation at 37 °C. However, the 1 % gelatine formulation presented comparable gelation profile with the CH hydrogel.

These results were confirmed by the time sweep experiments performed directly at 37 °C (Fig. 1b), which better represented the hydrogel behaviour when injected in the disc at body temperature. CH-gelatine hydrogels presented a rapid increase in  $G'$

with time at 37 °C. The addition of gelatine decreased the rate in a dose-dependent way, but gelation was rapid for all tested formulations (crossover of storage modulus  $G'$  and loss modulus  $G''$  in less than 15 s).

Interestingly, unconfined compressive properties (Fig. 1c,d) increased significantly in the presence of gelatine. The secant modulus, calculated at 15 % deformation [corresponding to physiological loading conditions (Johannessen *et al.*, 2006)], was significantly higher for the formulations with gelatine compared to control (Fig. 1d) ( $p < 0.05$ ). In addition, failure stress was significantly higher in the presence of gelatine compared to control ( $p < 0.0001$ ,  $p = 0.01$  and  $p = 0.02$  for 1, 1.5 and 2 % w/v gelatine, respectively), with the highest value obtained for 1 % w/v gelatine formulation (stress at failure =  $99 \pm 6$  kPa for control *vs.*  $142 \pm 12$  kPa for 1 % w/v gelatine). Hydrogel breakage occurred at similar deformation rate for all formulations ( $64 \pm 2$  %).

### Effect of gelatine on the viability and GAG production of cells encapsulated in CH hydrogels

As shown in Fig. 2, a high cell viability (between 89-92 %) was observed for all tested formulations at day 1, indicating no substantial cell death during NP encapsulation. Cell viability remained unchanged until day 14, indicating that gelatine addition did not affect NP cell survival. In fact, the DNA content

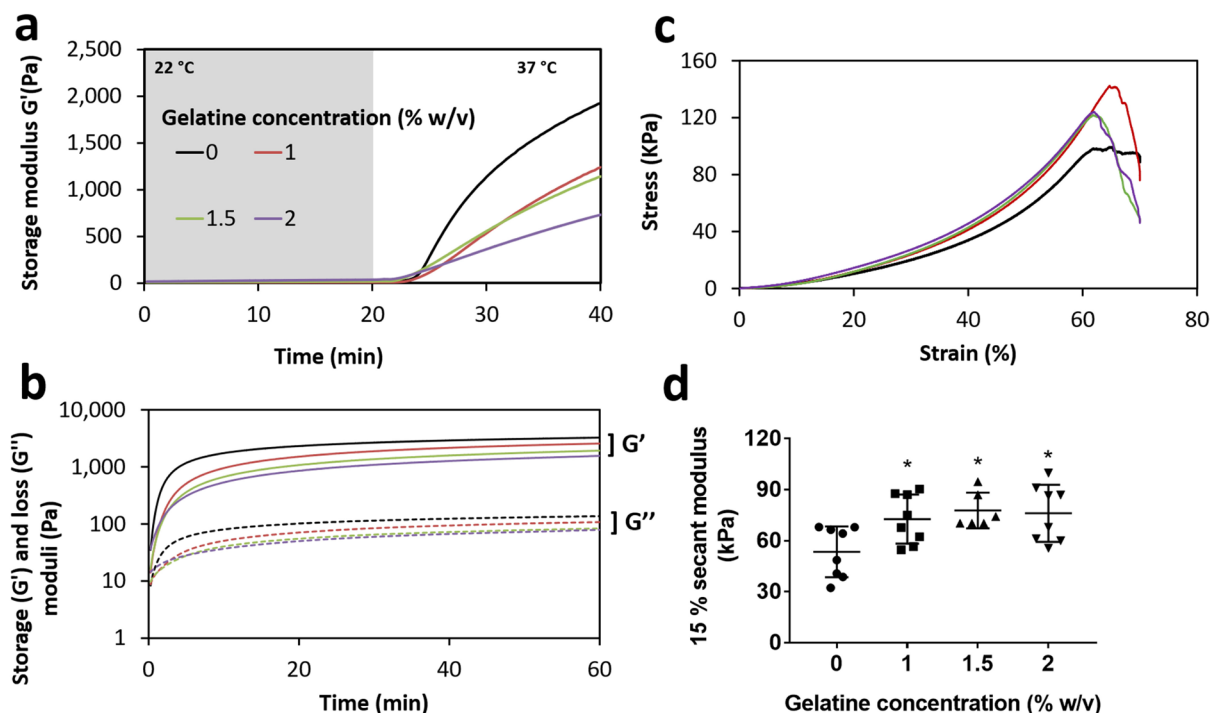
was also similar for all formulations after 14 d in culture (data not shown). However, NP cells after 14 d presented a spindle-like shape in CH-gelatine hydrogels, suggesting a better cell-matrix adhesion when compared to CH hydrogel (Fig. 2).

After 14 d in normal medium, total GAG synthesis (in the construct and released into the medium) was assessed. The results revealed that the NP cells encapsulated in CH-gelatine hydrogels produced a significantly higher GAG content after 14 d when compared to the control ( $p < 0.0001$ ; Fig. 3a). This increase was observed for all gelatine concentrations. GAG production per DNA (Fig. 3b) was also significantly higher ( $p < 0.01$ ) in the presence of gelatine, suggesting that gelatine increased cell activity.

Based on these results (NP cells responded similarly to all gelatine concentrations, but better mechanical and rheological properties were observed with 1 % gelatine), 1 % w/v gelatine was found to be the most appropriate concentration to supplement the CH hydrogel with. Thus, this formulation (CH-1 % gelatine) was selected for all following experiments.

### Diffusion of LN within the hydrogel

To evaluate the effect of LN on encapsulated cells, it is important to first verify that LN would diffuse through the CH hydrogel. To test this, CH hydrogel



**Fig. 1. Effect of gelatine concentration on rheological and unconfined compression properties of CH hydrogels.** (a,b) Time sweep experiments of CH hydrogel solutions as a function of gelatine concentration (0, 1, 1.5, and 2 % w/v). (a) Evolution of storage modulus with time at 22 °C (to confirm stability at room temperature) followed by temperature increase to 37 °C (strain 1 %, frequency 1 Hz, data shown as mean,  $n = 3$ ). (b) Evolution of storage modulus with time at 37 °C (strain 5 %, frequency 1 Hz, data shown as mean,  $n = 3$ ). (c,d) Unconfined compression. (c) Mean stress *versus* strain curves (up to 70 % of deformation at 100 %/min,  $n = 4$ ). (d) Secant modulus of hydrogels at 15 % deformation (mean  $\pm$  SD  $n = 8$ , \*  $p < 0.05$  compared to control). Tests were performed on cylindrical samples (14 mm diameter, 9 mm height) left to gel for 24 h at 37 °C.

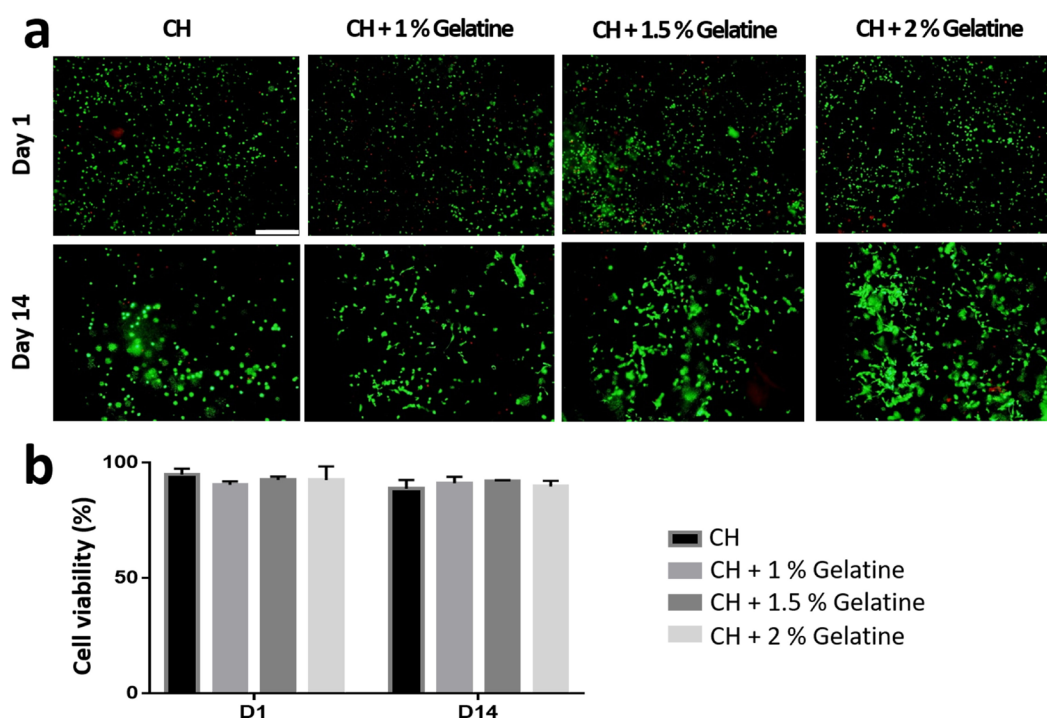
with or without gelatine was incubated in culture medium supplemented with fluorescent LN (with N terminal 5-FAM). The rapid diffusion of the fluorescent peptide in the gel confirmed that LN rapidly reached the encapsulated cells (Fig. 4). After 24 h, LN concentration was close to equilibrium ( $71 \mu\text{g/mL}$ ) in both CH ( $67 \pm 5 \mu\text{g/mL}$ ) and CH/1 % gelatine ( $67 \pm 7 \mu\text{g/mL}$ ) hydrogels.

### Effect of LN on GAG production by cells encapsulated in CH hydrogel

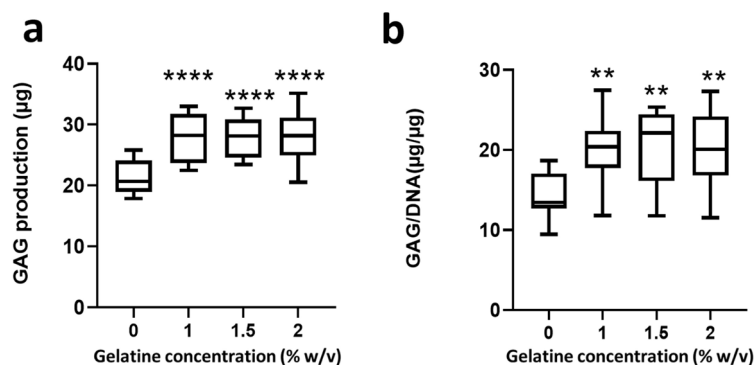
To evaluate the potential of LN to promote proteoglycan content in hydrogels, NP cells encapsulated in CH hydrogel (without gelatine) were

incubated in either normal medium or degenerative medium, mimicking healthy or degenerated disc environments, respectively. NP cells encapsulated in CH hydrogel were exposed to 10, 25 and  $100 \mu\text{g/mL}$  LN or medium alone for 14 d. GAG production and GAG/DNA ratio were assessed as a function of LN concentration and culture medium (Fig. 5).

As expected, in the degenerative medium, GAG synthesis by NP cells was 3-fold decreased compared with NP cells incubation in normal medium ( $p = 0.003$ ). This significant difference could be explained by the lower cell viability observed in the degenerative ( $56 \pm 8 \%$ ) compared to normal medium ( $81 \pm 3 \%$ ) (data not shown).



**Fig. 2. Viability of NP cells encapsulated in CH and CH-gelatin hydrogels.** (a) Live/dead assay performed after 1 or 14 d in culture. No significant differences were observed between conditions in terms of cell viability. Scale bar: 300  $\mu\text{m}$  (green for living cells and red for dead cells). (b) Percentage of viable cells according to cell counting on LIVE/DEAD<sup>TM</sup> images. Data are shown as mean  $\pm$  SD,  $n = 3$ .



**Fig. 3. GAG production by encapsulated NP cells in CH or in CH-gelatin hydrogels.** NP cells were encapsulated in CH or in CH-gelatin (1, 1.5 and 2 % w/v) hydrogels and cultured in normal medium for 14 d. (a) Total GAG production (in construct and released into the medium) was analysed by DMMB assay. (b) GAG production was normalised to DNA quantity (measured by PicoGreen<sup>®</sup> assay;  $n = 12-14$ , \*\*\*\*  $p < 0.0001$ , \*\*  $p < 0.01$ ).



LN did not elicit any significant effect on GAG content when constructs were cultured in normal medium (Fig. 5a,c). However, when cells were cultured in degenerative medium, the results were strikingly different, showing a statistically significant dose-dependent increase in proteoglycan content in response to LN supplementation, with maximal response at the highest dose of LN ( $p < 0.0001$ ) (Fig. 5b,d). Thus, when the degenerative medium was supplemented with 25  $\mu\text{g/mL}$  and 100  $\mu\text{g/mL}$  LN, GAG synthesis was increased by 3.1-fold and 4.7-fold, respectively, compared to the degenerative medium alone (Fig. 5b), reaching similar or higher levels of GAG synthesis compared to normal conditions. DNA content was similar for all LN concentrations (data not shown), suggesting that the increase in GAG production was due to increased cell activity, not cell proliferation. Indeed, similar trends were obtained when GAG production was normalised to DNA content (3.3-fold and 4.6-fold superior to the control) (Fig. 5d). However, no difference was observed at the low dose of LN (10  $\mu\text{g/mL}$ ).

#### Additive effect of LN and gelatine on GAG production in normal and degenerative environment

To determine the additive benefit of gelatine and LN on GAG production, NP cells encapsulated in CH or CH-1 % gelatine hydrogels were cultured for 14 d in the normal or degenerative medium supplemented with 100  $\mu\text{g/mL}$  LN or 50 ng/mL TGF- $\beta$ 1 (Fig. 6). TGF- $\beta$  was used as a positive control due to its confirmed role in promoting GAG production (Smith *et al.*, 2014).

Notwithstanding the medium used, TGF- $\beta$  significantly increased GAG synthesis in NP cells ( $p < 0.0001$ ) compared to the control (Fig. 6a,b). GAG production in the presence of TGF- $\beta$  was significantly more in CH-1 % gelatine compared to CH hydrogel,

in both normal (Fig. 6a,  $p = 0.02$ ) and degenerative medium (Fig. 6b,  $p < 0.0001$ ), suggesting a potential additive effect of TGF- $\beta$  and gelatine on NP cell activity. However, GAG synthesis in the presence of TGF- $\beta$  was reduced in the degenerative compared to normal medium by over 41 % and 30 % in the CH and CH-1 % gelatine hydrogels, respectively.

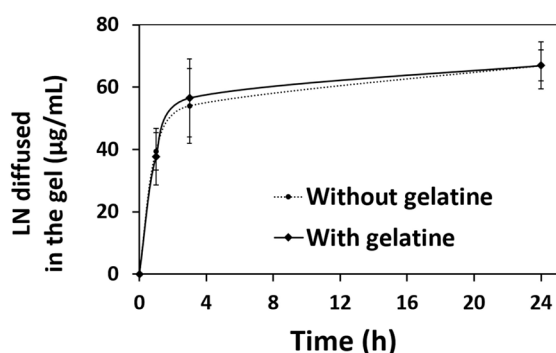
GAG concentrations in constructs cultured in normal medium supplemented with LN were similar to that in controls (Fig. 6a). In contrast, a significant increase was observed when incubated in degenerative medium ( $p < 0.0001$  for LN without gelatine;  $p = 0.003$  for LN with gelatine; Fig. 6b). Since there was no difference in DNA content between conditions after 14 d (data not shown), a similar trend was observed for GAG production per DNA (Fig. 6c,d). Interestingly, in degenerative medium, GAG production in the presence of LN was more than in normal medium ( $p = 0.0007$  for CH and CH-1 % gelatine hydrogels) and reached similar levels to that in the presence of TGF- $\beta$ . Gelatine addition to the hydrogel tended to further increase GAG production. This difference was significant in the degenerative ( $p = 0.03$ ) but not in normal medium. The observations were similar when analysing GAG production per DNA (GAG/DNA) in normal (Fig. 6c) and degenerative media (Fig. 6d). This indicated that in early disc degeneration, LN alone has the potential to restore proteoglycan content and that an additional benefit is achieved by including gelatine in the construct.

#### Cell viability of encapsulated NP cells supplemented with either LN or TGF- $\beta$

To verify that supplementation of 50 ng/mL TGF- $\beta$  or 100  $\mu\text{g/mL}$  LN in a normal or degenerative medium was not detrimental to the cells, cell viability was assessed. Cell viability was maintained at  $> 80$  % when NP cells were encapsulated in CH or CH-1 % gelatine hydrogels supplemented with either LN or TGF- $\beta$  in normal conditions after 14 d. In contrast, at day 14 (Fig. 7), the incubation in degenerative medium induced relatively high cellular death ( $52 \pm 11$  % viability) in CH hydrogel, indicating that the medium used in these studies mimicked the degenerative disc environment where cell death is commonly observed (Trout *et al.*, 1982). However, CH-1 % gelatine hydrogels incubated in the degenerative medium supplemented with LN ( $76 \pm 11$  %) showed greater cell viability compared to TGF- $\beta$  and control ( $50 \pm 12$  %), suggesting that LN may have a protective effect against cell death in the presence of gelatine.

#### Discussion

To the authors' knowledge, this was the first study evaluating an injectable therapy for IVD repair in conditions simulating the degenerative disc microenvironment. A thermosensitive injectable

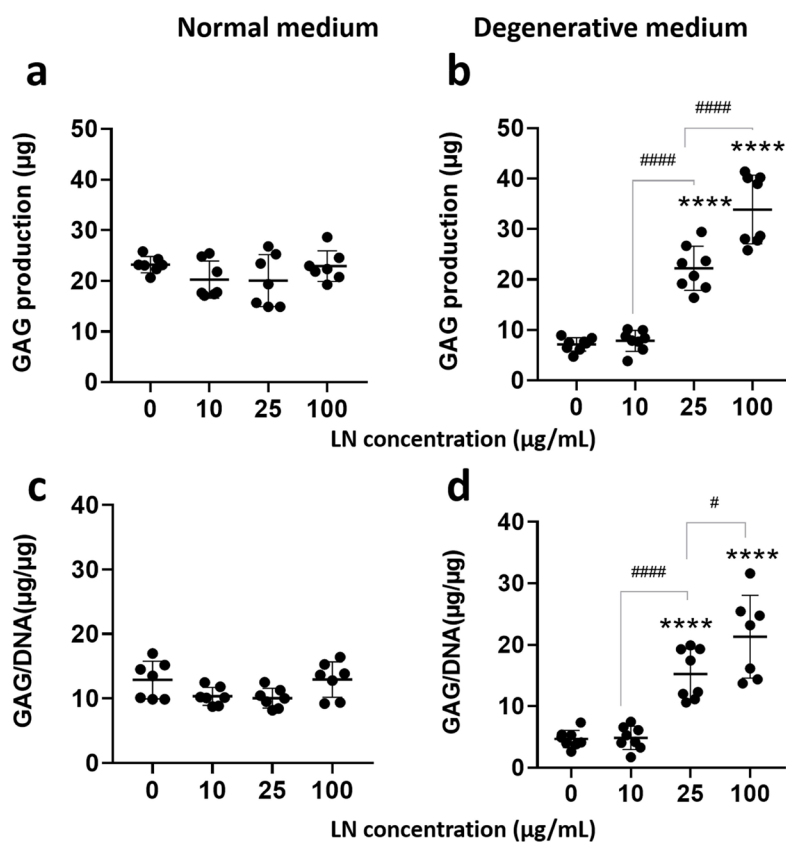


**Fig. 4. LN diffusion within the CH and CH-1 % gelatine hydrogels.** 5-FAM-conjugated LN was placed on top of the CH hydrogels with or without gelatine. The medium was retrieved and analysed spectrophotometrically to quantify the remaining LN in the medium at each time point. This amount was subtracted from the initial LN content to assess the amount of LN that diffused into the gel. LN concentration in the gel after 1, 3 and 24 h are shown as mean  $\pm$  SD,  $n = 4-6$ .

CH hydrogel was previously developed, showing adequate mechanical properties and cell survival for IVD repair but a suboptimal synthesis of proteoglycans (Alinejad *et al.*, 2019). Since the loss of proteoglycans is one of the most notable changes in IVD degeneration, the primary goal of this investigation was to evaluate the potential of LN and gelatine to increase proteoglycan production by encapsulated NP cells in CH hydrogels, in a degenerative IVD microenvironment.

Data indicated that CH hydrogel supplemented with 1 % w/v gelatine increased significantly GAG production by NP cells while showing adequate gelation profile and a significant increase in compressive mechanical properties. Gelatine has been already used by several researchers in combination with CH to form 2D membranes or 3D scaffolds for tissue engineering. It has been used either to form polyelectrolytes with CH (Huang *et al.*, 2005; Mao *et al.*, 2003; Ng *et al.*, 2016) or to reinforce CH-BGP gels with limited mechanical properties (Cheng *et al.*, 2010). Cheng *et al.* (2010) showed that 1 % w/v gelatine decreases the gelation time of CH-BGP gels from more than 10 min to less than 1 min and increases their storage modulus from < 10 Pa to ~ 500 Pa after 10 min at 37 °C. In the present study, CH was mixed with SHC and BGP as gelling agents,

to increase drastically the mechanical properties and shorten gelation time, while allowing for better cytocompatibility due to the lower BGP content (Ceccaldi *et al.*, 2017). As expected, results confirmed larger  $G'$  values at 10 min (1,700 and 1,300 Pa for CH and CH-1 % gelatine, respectively) and a shorter gelation time for all CH-gelatin formulations (< 15 s at 37 °C). Results were also in agreement with Cheng *et al.* (2010) with respect to increased mechanical properties in the presence of gelatine. However, gelatine tended to reduce the gelation rate (as shown by slower increase in  $G'$  and lower  $G'$  after 1 h at 37 °C). This result may be explained by gelatine charge. Indeed, at physiological pH, both gelatine type A and CH molecules are positively charged (Van Vlierberghe *et al.*, 2014). The mechanism of CH thermogelation is related to CH chain's neutralisation by BGP and SHC, followed by proton transfer when increasing the temperature (Lavertu *et al.*, 2008). The presence of positively charged gelatine in the solution may have reduced the degree of neutralisation of CH chains and delay the proton transfer to the base. This may explain why after 1 h the viscoelastic properties of CH-gelatin hydrogels were lower than those of CH hydrogel. However, the compressive properties of CH-gelatin hydrogels after 24 h gelation were improved, especially the mechanical strength, which



**Fig. 5. Proteoglycan (GAG) production by encapsulated NP cells treated with LN.** Bovine NP cells were encapsulated in CH hydrogels and cultured in either normal or degenerative medium in the presence of LN (10, 25 and 100 µg/mL) or medium without peptide supplementation for 14 d. GAG content was taken as a measurement of proteoglycan. Total GAG production per construct in (a) normal and (b) degenerative media. GAG production per 1 µg DNA in (c) normal and (d) degenerative media. Data are expressed as mean  $\pm$  SD, one-way ANOVA followed by Tukey *post-hoc* test,  $n = 7$ , \*\*\*\*  $p < 0.0001$  (compared to control).

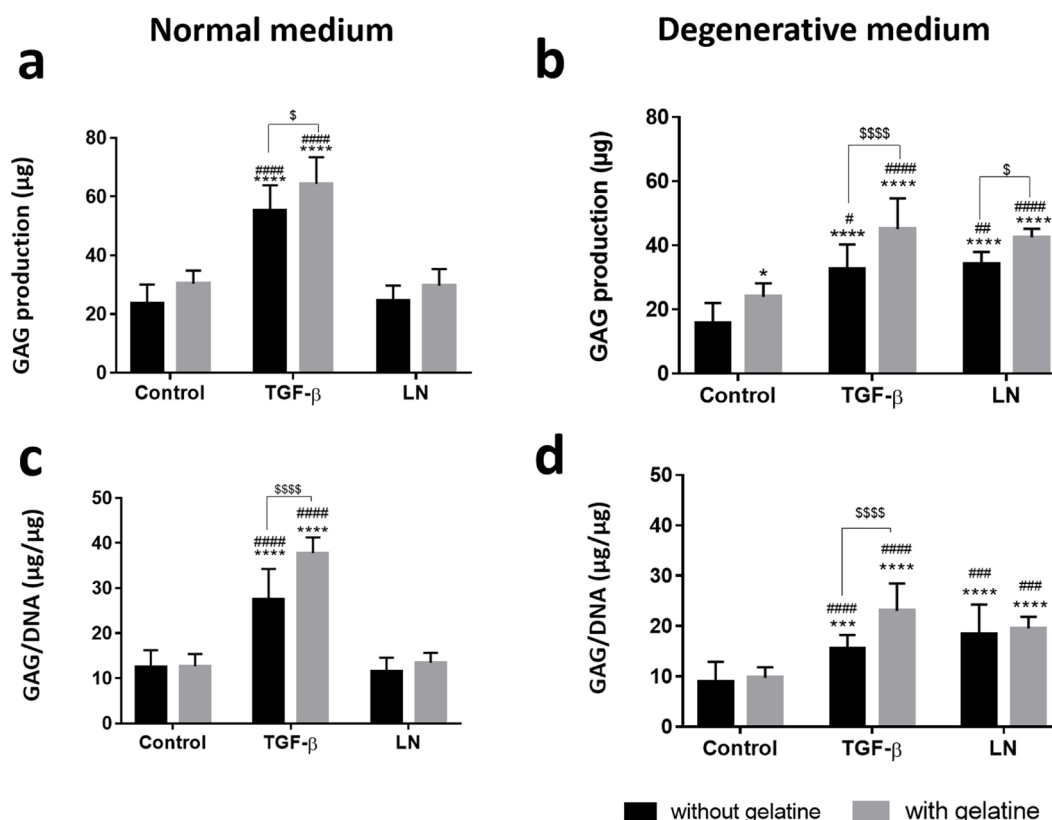


increased from 99 to 142 kPa (with 1 % gelatine). Gelatine chains are able to form hydrogen bonds when mixed together (Cheng *et al.*, 2003; Ng *et al.*, 2016). Thus, gelatine addition may have transformed CH hydrogel from a simple physical hydrogel network to a reinforced IPN (Karak, 2012). Alinejad *et al.* (2019) showed that CH hydrogel presents compressive properties very similar to human NP. In the present study, the rigidity in compression was slightly increased by the addition of gelatine, but remained in the same range, which allowed us to conclude that the CH-gelatin hydrogels also presented compressive properties close to human NP.

In addition to this mechanical benefit, gelatine induced more GAG production by encapsulated NP cells. As shown in the live/dead images, CH hydrogels supplemented with gelatine increased NP cells adhesion to the scaffold. Indeed, gelatine provides amino acid sequences similar to collagen to help facilitate cell adhesion (Huang *et al.*, 2005; Zheng *et al.*, 2018), which can stimulate GAG production. Besides, through some structural changes in the hydrogel, gelatine may have increased the amount of nutrient and oxygen inside the gel, which can be also translated into an increase in GAG production (Xia *et al.*, 2004).

TGF- $\beta$  is among the several growth factors, including GDF-5 and OP-1, that have been previously investigated and showed promising results in restoring proteoglycan content and stimulating new disc matrix formation (Masuda and An, 2004). Although these strategies have reached animal studies and been approached in the clinic (Web ref. 2), some may have delayed or reduced pain, but none have achieved biological repair of a diseased IVD (Hodgkinson *et al.*, 2019). TGF- $\beta$  is one of the most potent growth factors for promoting matrix production. Activation of TGF- $\beta$  plays a critical role in mechanical signalling transduction to regulate IVD cell function and homeostasis (Bian *et al.*, 2017). TGF- $\beta$  can increase GAG production through increased mRNA expression and inhibition of proteases that induce ECM protein degradation (Bassols and Massague, 1988; Roberts *et al.*, 1992). However, TGF- $\beta$  is not suitable for clinical applications due to its tumorigenicity (Brahmkhatri *et al.*, 2015) and high cost. It was used in the present study only as a positive control.

Compared to other growth factors, known for their involvement in osteogenesis and unwanted and uncontrolled side effects (Lebrun, 2012), LN action is specific to chondrogenic tissues such as IVD and



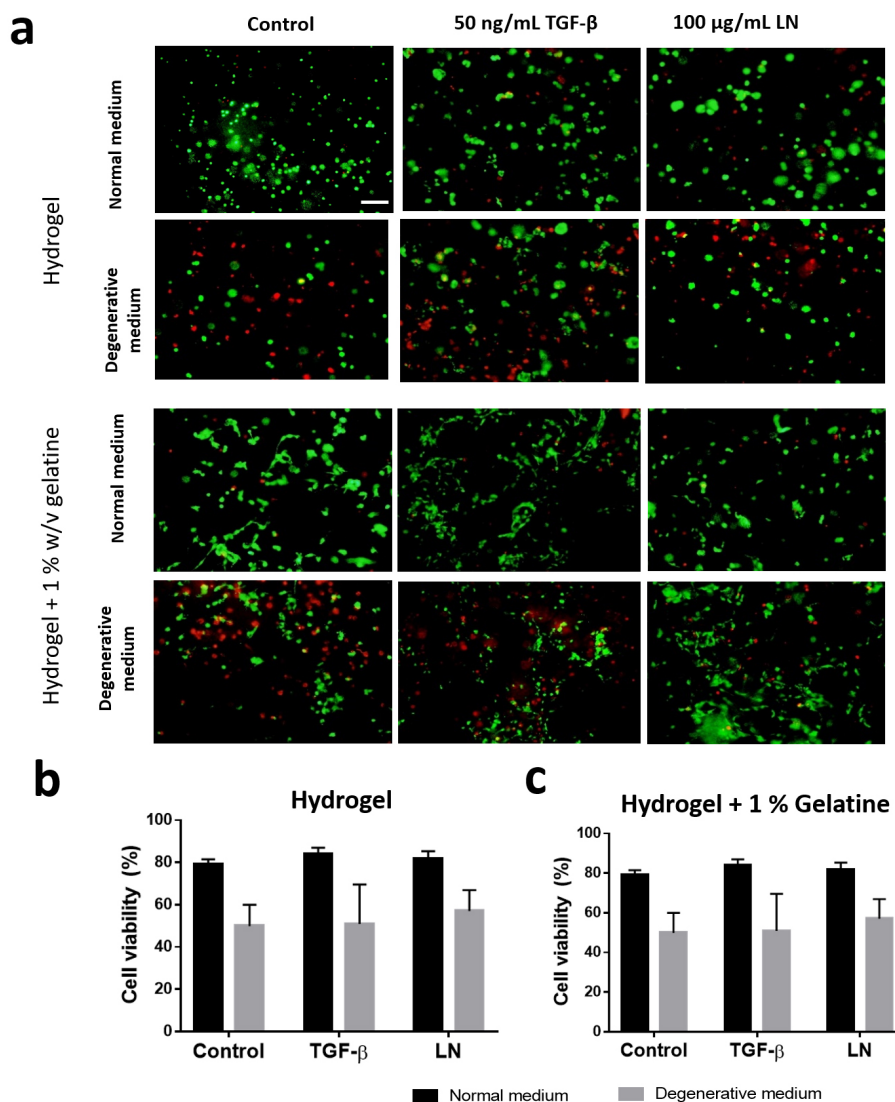
**Fig. 6. Proteoglycan production by encapsulated NP treated with either LN or TGF- $\beta$ .** NP cells were seeded in CH or CH-1 % gelatine hydrogel and cultured in (a,c) normal or (b,d) degenerative media in the presence of 100  $\mu$ g/mL LN, 50 ng/mL TGF- $\beta$  or medium without peptide supplementation for 14 d. GAG content was taken as a measurement of proteoglycan. Data shown as mean  $\pm$  SD, two-way ANOVA followed by Tukey *post-hoc* comparison (\*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$  compared to control without gelatine, \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  compared to control with gelatine, \$  $p < 0.05$ , \$\$\$  $p < 0.001$ , \$\$\$\$  $p < 0.0001$ ,  $n = 8-16$ ).

cartilage (Mwale *et al.*, 2014). Previous studies have demonstrated that LN acts as an anabolic agent in the IVD, increasing the expression and synthesis of structural matrix proteins and reversing the structural deterioration (Antoniou *et al.*, 2012; Bach *et al.*, 2017; Gawri *et al.*, 2012; Mwale *et al.*, 2011). It inhibits both the expression of pro-inflammatory cytokines and pain-associated factors (Noorwali *et al.*, 2018), and the co-administration of MSCs and LN synergistically enhances the repair of IVDs in a disc degeneration model (Mwale *et al.*, 2014). Therefore, LN meets the criteria for an ideal IVDD treatment, reversing structural degradation of the IVD and blocking the inflammatory and pain-producing pathways.

Most of the studies testing the effect of bioactive agents on NP cells were conducted either in chemically controlled medium (Smith *et al.*, 2014) or medium simulating normal culture conditions (Gawri *et al.*, 2012; Masuda, 2008; Miyamoto *et*

*al.*, 2006). In fact, the IVD represents a very harsh microenvironment even in its healthy state (low oxygen levels, high osmolarity, nutritional deficits and high mechanical loading). During degeneration, these conditions are further aggravated by numerous factors such as nutritional deficits, increased matrix acidity and inflammation, which can affect cell survival, behaviour and ECM production. The degenerative medium used in the present study, by composition, mimicked closely the degenerative IVD environment. The medium had a lower pH and osmolarity and a selectively higher mineral content, including supplementation with the inflammatory cytokine IL-1 $\beta$ , representing the conditions of a degenerative disc (van Dijk *et al.*, 2011), which was targeted by the present therapy.

Data indicated that, under conditions mimicking the degenerative disc environment, LN induced a strong increase in GAG production by NP cells.



**Fig. 7.** Effect of LN or TGF- $\beta$  supplementation on bovine NP cell viability encapsulated in CH or CH-1 % gelatine scaffolds. (a) LIVE/DEAD<sup>TM</sup> assay performed at 14 d on NP cells encapsulated in hydrogel with or without gelatine in normal and degenerative medium alone (control), supplemented with 50 ng/mL TGF- $\beta$  or 100  $\mu$ g/mL LN. Scale bar: 200  $\mu$ m. Green for living cells and red for dead cells. (b,c) Percentage of viable cells according to cell counting on LIVE/DEAD<sup>TM</sup> images. Data are shown as mean  $\pm$  SD,  $n = 3$ , two-way ANOVA followed by Tukey *post-hoc* comparison. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

This finding was in an agreement with earlier data (Alaqeel *et al.*, 2020) showing that LN can restore GAG production to healthy control levels in human osteoarthritic cartilage when exposed to IL-1 $\beta$ , an inflammatory cytokine known for its catabolic activities in cartilage and IVD (Masuda and An, 2004; Yang *et al.*, 2015). The strong effect of LN observed in these conditions could be partly explained by the fact that LN inhibits IL-1 $\beta$  signalling pathways (Alaqeel *et al.*, 2020). Interestingly, the study's findings demonstrated that in a degenerative medium, the level of GAG achieved with LN reached similar levels to those observed with TGF- $\beta$ . However, LN did not stimulate GAG production in normal medium. Previous work has also demonstrated that LN has no apparent effect in the medial tibial plateau of a rabbit model of osteoarthritis (Antoniou *et al.*, 2019). The authors reported that this may be related to the low-grade of cartilage pathology occurring at this site in the model. This observation raises the possibility that LN exerts its influence once matrix degradation exceeds turnover (Alaqeel *et al.*, 2020; Antoniou *et al.*, 2019). In contrast, TGF- $\beta$  increased GAG production in both normal and degenerative conditions.

Interestingly, NP cell survival appeared to be stimulated by LN in the presence of gelatine in degenerative medium. In contrast, supplementation with TGF- $\beta$  did not affect cell viability in a similar manner. Antoniou *et al.* (2019) demonstrated that LN has chondroprotective properties that may have been enhanced in the presence of gelatine, protecting NP cells against the harmful composition of the degenerative medium. Furthermore, in a degenerative environment, LN and gelatine induced more GAG production compared to LN and gelatine used separately, suggesting an independent additive benefit. This could be explained by the fact that LN and gelatine do not affect the cells in the same manner. Gelatine improves GAG production by inducing a change in cell adhesion and organisation while LN improves GAG synthesis through stimulation of Sox9 expression and inhibition of inflammatory protein (Antoniou *et al.*, 2012).

Despite the significant benefit of gelatine and LN, the amount of synthesised GAG was still lower (10 %) than in healthy human disc (Alini *et al.*, 2003). In further work, a prolonged incubation time, an increase in cells density in the gel and a mechanical stimulation during the culture may help increase GAG production close to *in vivo* levels.

The present study established the proof-of-concept that LN embedded in CH or CH-gelatine scaffolds could increase the activity of encapsulated cells. Further investigations are required to define how exactly LN should be incorporated in the gel for clinical transfer. The first strategy may be to add LN in the cell suspension before encapsulation. Then, rapid diffusion of LN is expected but should not be an issue *in vivo* since the released LN will stay in the disc and may stimulate native disc cells surrounding the injected scaffold. Another option may be to embed

LN in nanoparticles before encapsulation to better control the release profile.

This study was performed with NP cells to determine the effectiveness of the technology. Next, *ex vivo* and *in vivo* steps will be performed to validate the findings in a clinically available cell source such as human stem cells, to facilitate the translation of the technology.

## Conclusions

LN and gelatine were evaluated as bioactive agents in order to establish whether they were capable of increasing the viability and activity of NP cells encapsulated in 3D CH thermosensitive hydrogels. LN increased GAG production in a degenerative medium to the same level as TGF- $\beta$ . Addition of 1 % gelatine in a CH hydrogel further increased GAG production and interestingly improved the mechanical resistance of a CH hydrogel, whose elasticity was similar to native NP tissue. The results supported the concept that a CH hydrogel supplemented with LN and gelatine may be an ideal scaffold for cell-based supplementation to help enhance the function of the NP during the early stages of IVD degeneration.

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## References

- Alaqeel M, Grant M, Epure L, Salem O, AlShaer A, Huk O, Bergeron S, Zukor D, Kc R, Im H (2020) Link N suppresses interleukin-1 $\beta$ -induced biological effects on human osteoarthritic cartilage. *Eur Cell Mater* **39**: 65-76.
- Alinejad Y, Adoungotchodo A, Grant MP, Epure LM, Antoniou J, Mwale F, Lerouge S (2019) Injectable chitosan hydrogels with enhanced mechanical properties for nucleus pulposus regeneration. *Tissue Eng Part A* **25**: 303-313.
- Alini M, Li W, Markovic P, Aebi M, Spiro RC, Roughley PJ (2003) The potential and limitations of a cell-seeded collagen/hyaluronan scaffold to engineer an intervertebral disc-like matrix. *Spine (Phila Pa 1976)* **28**: 446-454.
- Antoniou J, Epure L, Grant M, Richard H, Sampalis J, Roughley P, Laverty S, Mwale F (2019) Short link N acts as a disease modifying osteoarthritis drug. *Eur Cell Mater* **37**: 347-359.



- Antoniou J, Wang HT, Alaseem AM, Haglund L, Roughley PJ, Mwale F (2012) The effect of Link N on differentiation of human bone marrow-derived mesenchymal stem cells. *Arthritis Res Ther* **14**: R267. DOI: 10.1186/ar4113.
- Assaad E, Maire M, Lerouge S (2015) Injectable thermosensitive chitosan hydrogels with controlled gelation kinetics and enhanced mechanical resistance. *Carbohydr Polym* **130**: 87-96.
- Bach FC, Laagland LT, Grant MP, Creemers LB, Ito K, Meij BP, Mwale F, Tryfonidou MA (2017) Link-N: the missing link towards intervertebral disc repair is species-specific. *PLoS One* **12**: e0187831. DOI: 10.1371/journal.pone.0187831.
- Bassols A, Massague J (1988) Transforming growth factor beta regulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. *J Biol Chem* **263**: 3039-3045.
- Bian Q, Ma L, Jain A, Crane JL, Kebaish K, Wan M, Zhang Z, Edward Guo X, Sponseller PD, Séguin CA, Riley LH, Wang Y, Cao X (2017) Mechanosignaling activation of TGF $\beta$  maintains intervertebral disc homeostasis. *Bone Res* **5**: 17008. DOI: 10.1038/boneres.2017.8.
- Brahmkhatri VP, Prasanna C, Atreya HS (2015) Insulin-like growth factor system in cancer: novel targeted therapies. *Biomed Res Int* **2015**: 538019-538019.
- Ceccaldi C, Assaad E, Hui E, Buccionyte M, Adoungotchodo A, Lerouge S (2017) Optimization of injectable thermosensitive scaffolds with enhanced mechanical properties for cell therapy. *Macromol Biosci* **17**. DOI: 10.1002/mabi.20160043.
- Cheng M, Deng J, Yang F, Gong Y, Zhao N, Zhang X (2003) Study on physical properties and nerve cell affinity of composite films from chitosan and gelatine solutions. *Biomaterials* **24**: 2871-2880.
- Cheng Y-H, Yang S-H, Su W-Y, Chen Y-C, Yang K-C, Cheng WT-K, Wu S-C, Lin F-H (2010) Thermosensitive chitosan-gelatine-glycerol phosphate hydrogels as a cell carrier for nucleus pulposus regeneration: an *in vitro* study. *Tissue Eng Part A* **16**: 695-703.
- Freemont A (2009) The cellular pathobiology of the degenerate intervertebral disc and discogenic back pain. *Rheumatology (Oxford)* **48**: 5-10.
- Frith JE, Cameron AR, Menzies DJ, Ghosh P, Whitehead DL, Gronthos S, Zannettino ACW, Cooper-White JJ (2013) An injectable hydrogel incorporating mesenchymal precursor cells and pentosan polysulphate for intervertebral disc regeneration. *Biomaterials* **34**: 9430-9440.
- Garnjanagoonchorn W, Wongekalak L, Engkagul A (2007) Determination of chondroitin sulfate from different sources of cartilage. *Chemical Engineering and Processing: Process Intensification* **46**: 465-471.
- Gawri R, Antoniou J, Ouellet J, Awwad W, Steffen T, Roughley P, Haglund L, Mwale F (2012) Best paper NASS 2013: link-N can stimulate proteoglycan synthesis in the degenerated human intervertebral discs. *Eur Cell Mater* **26**: 107-119.
- Gawri R, Ouellet J, Önnarfjord P, Alkhatib B, Steffen T, Heinegård D, Roughley P, Antoniou J, Mwale F, Haglund L (2014) Link N is cleaved by human annulus fibrosus cells generating a fragment with retained biological activity. *J Orthop Res* **32**: 1189-1197.
- Gentile P, Nandagiri VK, Daly J, Chiono V, Mattu C, Tonda-Turo C, Ciardelli G, Ramtools Z (2016) Localised controlled release of simvastatin from porous chitosan-gelatine scaffolds engrafted with simvastatin loaded PLGA-microparticles for bone tissue engineering application. *Mater Sci Eng C Mater Biol Appl* **59**: 249-257.
- Gilchrist CL, Chen J, Richardson WJ, Loeser RF, Setton LA (2007) Functional integrin subunits regulating cell-matrix interactions in the intervertebral disc. *J Orthop Res* **25**: 829-840.
- Gilchrist CL, Darling EM, Chen J, Setton LA (2011) Extracellular matrix ligand and stiffness modulate immature nucleus pulposus cell-cell interactions. *PLoS One* **6**: e27170. DOI: 10.1371/journal.pone.0027170.
- Hodgkinson T, Shen B, Diwan A, Hoyland JA, Richardson SM (2019) Therapeutic potential of growth differentiation factors in the treatment of degenerative disc diseases. *JOR Spine* **2**: e1045. DOI: 10.1002/jsp2.1045.
- Huang Y, Onyeri S, Siewe M, Moshfeghian A, Madhally SV (2005) *In vitro* characterization of chitosan-gelatine scaffolds for tissue engineering. *Biomaterials* **26**: 7616-7627.
- Iatridis JC, Nicoll SB, Michalek AJ, Walter BA, Gupta MS (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.
- Jaipan P, Nguyen A, Narayan RJ (2017) Gelatine-based hydrogels for biomedical applications. *MRS Commun* **7**: 416-426.
- Johannessen W, Auerbach JD, Wheaton AJ, Kurji A, Borthakur A, Reddy R, Elliott DM (2006) Assessment of human disc degeneration and proteoglycan content using T1 $\rho$ -weighted magnetic resonance imaging. (*Phila Pa* 1976) **31**: 1253-1257.
- Karak N (2012) Fundamentals of polymers. In: *Vegetable oil-based polymers*. Woodhead Publishing, pp: 1-30.
- Kim DJ, Moon SH, Kim H, Kwon UH, Park MS, Han KJ, Hahn SB, Lee HM (2003) Bone morphogenetic protein-2 facilitates expression of chondrogenic, not osteogenic, phenotype of human intervertebral disc cells. *Spine (Phila Pa 1976)* **28**: 2679-2684.
- Lavertu M, Fillion D, Buschmann MD (2008) Heat-induced transfer of protons from chitosan to glycerol phosphate produces chitosan precipitation and gelation. *Biomacromolecules* **9**: 640-650.
- Lebrun J-J (2012) The dual role of TGF $\beta$  in human cancer: from tumor suppression to cancer metastasis. *ISRN Mol Biol* **2012**: 381428-381428.
- Lee BH, Shirahama H, Cho N-J, Tan LP (2015) Efficient and controllable synthesis of highly

substituted gelatine methacrylamide for mechanically stiff hydrogels. *RSC Advances* **5**: 106094-106097.

Liang HC, Chang WH, Liang HF, Lee MH, Sung HW (2004) Crosslinking structures of gelatin hydrogels crosslinked with genipin or a water-soluble carbodiimide. *J Appl Polym Sci* **91**: 4017-4026.

Mao JS, Zhao LG, Yin YJ, Yao KD (2003) Structure and properties of bilayer chitosan-gelatin scaffolds. *Biomaterials* **24**: 1067-1074.

Masuda K (2008) Biological repair of the degenerated intervertebral disc by the injection of growth factors. *Eur Spine J* **17**: 441. DOI: 10.1007/s00586-008-0749-z.

Masuda K, An HS (2004) Growth factors and the intervertebral disc. *Spine J* **4**: 330S-340S.

Melching L, Roughley P (1985) The role of link protein in mediating the interaction between hyaluronic acid and newly secreted proteoglycan subunits from adult human articular cartilage. *J Biol Chem* **260**: 16279-16285.

Miyamoto K, Masuda K, Kim JG, Inoue N, Akeda K, Andersson GB, An HS (2006) Intradiscal injections of osteogenic protein-1 restore the viscoelastic properties of degenerated intervertebral discs. *Spine J* **6**: 692-703.

Mwale F, Masuda K, Pichika R, Epure LM, Yoshikawa T, Hemmad A, Roughley PJ, Antoniou J (2011) The efficacy of Link N as a mediator of repair in a rabbit model of intervertebral disc degeneration. *Arthritis Res Ther* **13**: R120. DOI: 10.1186/ar3423.

Mwale F, Wang HT, Roughley P, Antoniou J, Haglund L (2014) Link N and mesenchymal stem cells can induce regeneration of the early degenerate intervertebral disc. *Tissue Eng Part A* **20**: 2942-2949.

Ng WL, Yeong WY, Naing MW (2016) Development of polyelectrolyte chitosan-gelatin hydrogels for skin bioprinting. *Procedia CIRP* **49**: 105-112.

Nguyen TH, Randolph DC, Talmage J, Succop P, Travis R (2011) Long-term outcomes of lumbar fusion among workers' compensation subjects: a historical cohort study. *Spine (Phila Pa 1976)* **36**: 320-331.

Noorwali H, Grant MP, Epure LM, Madiraju P, Sampen H-J, Antoniou J, Mwale F (2018) Link N as a therapeutic agent for discogenic pain. *JOR Spine* **1**: e1008-e1008.

O'Halloran DM, Pandit AS (2007) Tissue-engineering approach to regenerating the intervertebral disc. *Tissue Eng* **13**: 1927-1954.

Raj PP (2008) Intervertebral disc: anatomy-physiology-pathophysiology-treatment. *Pain Pract* **8**: 18-44.

Roberts AB, McCune BK, Sporn MB (1992) TGF- $\beta$ : regulation of extracellular matrix. *Kidney Int* **41**: 557-559.

Shapiro IM, Risbud MV (2013) The intervertebral disc: molecular and structural studies of the disc in health and disease. Springer-Verlag Wien. DOI: 10.1007/978-3-7091-1535-0.

Smith LJ, Gorth DJ, Showalter BL, Chiaro JA, Beattie EE, Elliott DM, Mauck RL, Chen W, Malhotra NR (2014) *In vitro* characterization of a stem-cell-

seeded triple-interpenetrating-network hydrogel for functional regeneration of the nucleus pulposus. *Tissue Eng Part A* **20**: 1841-1849.

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.

Trout JJ, Buckwalter JA, Moore KC (1982) Ultrastructure of the human intervertebral disc: II. Cells of the nucleus pulposus. *Anat Rec* **204**: 307-314.

van Dijk B, Potier E, Ito K (2011) Culturing bovine nucleus pulposus explants by balancing medium osmolarity. *Tissue Eng Part C Methods* **17**: 1089-1096.

Van Vlierberghe S, Graulus GJ, Keshari Samal S, Van Nieuwenhove I, Dubrue P (2014) Porous hydrogel biomedical foam scaffolds for tissue repair. In: *Biomedical foams for tissue engineering applications*. Editor: Netti PA. Woodhead Publishing. pp: 335-390.

Wu PH, Kim HS, Jang I-T (2020) Intervertebral disc diseases part 2: a review of the current diagnostic and treatment strategies for intervertebral disc disease. *Int J Mol Sci* **21**: 2135. DOI: 10.3390/ijms21062135.

Xia W, Liu W, Cui L, Liu Y, Zhong W, Liu D, Wu J, Chua K, Cao Y (2004) Tissue engineering of cartilage with the use of chitosan-gelatin complex scaffolds. *J Biomed Mater Res B Appl Biomater* **71**: 373-380.

Yang W, Yu X-H, Wang C, He W-S, Zhang S-J, Yan Y-G, Zhang J, Xiang Y-X, Wang W-J (2015) Interleukin-1 $\beta$  in intervertebral disk degeneration. *Clin Chim Acta* **450**: 262-272.

Zheng Y, Liang Y, Zhang D, Sun X, Liang L, Li J, Liu Y-N (2018) Gelatin-based hydrogels blended with gellan as an injectable wound dressing. *ACS Omega* **3**: 4766-4775.

## Web References

1. Idataresearch.com [19-04-2021]
2. <https://clinicaltrials.gov/NCT01182337> [19-04-2021]

## Discussion with Reviewers

**Zhen Li:** What are the main obstacles using the technique described in the present study for NP clinical regeneration?

**Authors:** The lack of therapeutic approaches in development for this under-served and growing market represents a significant opportunity for this technology. Furthermore, there are several distinct clinical indications within IVDD for the technology to be used either as a prophylactic/preventative stand-alone therapy or as an adjunctive therapy to surgical intervention. The main obstacles for NP clinical regeneration have been that we have mainly been reliant on academic research and that there has been a lack of pharmaceutical peptide development.

Recently, we have started to industrialise the science and focus on drug development by availing ourselves of the adMare (adMare BioInnovations, Vancouver, Canada) capabilities and infrastructure. This will help achieving key preclinical development milestones to support investigational new drug (IND)-enabling studies, first-in-human (FIH) clinical trials and financing.

**Svenja Illien-Junger:** You demonstrated that NP-cells-seeded CH-gelatine + LN hydrogels maintain cell viability and increase GAG production under degenerative conditions, which you suggested was due to the inhibitory role of LN on IL-1 $\beta$  pathways.

Would you expect a similar protective effect of LN on cell viability and GAG production by supplementing the degenerative medium with pro-inflammatory mediators such as lipopolysaccharide or TNF- $\alpha$ ?

**Authors:** Although LN can inhibit the signalling of IL-1, preliminary data suggested it had less effect on TNF- $\alpha$ . Studies are underway to understand the protective effect of LN on cell viability and ECM production when degenerative medium is supplemented with lipopolysaccharide.

**Editor's note:** The Scientific Editor responsible for this paper was Mauro Alini.