Thermally Triggered Injectable Hydrogel Which Induces Mesenchymal Stem Cell Differentiation To Promote Regeneration Of The Intervertebral Disc

Abbey A. Thorpe, BSc1, Victoria Boyes, BSc, PhD2, Chris Sammon, BSc, PhD2, Christine L. Le Maitre, BSc PhD1.
1Biomedical Research Centre, Sheffield Hallam University, Sheffield, United Kingdom, 2Materials and Engineering Research Institute, Sheffield Hallam University, Sheffield, United Kingdom.

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Introduction: Instability of the motion segment as a result of intervertebral disc (IVD) degeneration is well known as a major cause of low back pain (Balague et al 2011). Degeneration in the central nucleus pulposus (NP) is characterised by a loss of extracellular matrix (ECM) components including proteoglycans particularly aggrecan, and a switch in collagen synthesis from collagen type II to collagen type I, thus resulting in a more fibrous NP tissue with an overall decreased disc height (Boos et al 1997). The majority of therapies currently in practice for IVD degeneration attempt to relieve patient symptoms by fusion or removal of the disc tissue with no attempt at regeneration (Lund and Oxland 2011). A number of tissue engineering and stem cell approaches have been investigated to restore disc height and simultaneously regenerate the degenerate IVD. However, many of the proposed therapies would entail lengthy/risky operations to 'implant' the tissue regeneration scaffold. In this work we aim to develop a novel, injectable biomaterial delivery system which can deliver mesenchymal stem cells (MSCs) to the degenerate nucleus pulposus (NP) to stimulate regeneration, together with inhibitors of degeneration, if required, and provide mechanical support to the motion segment enabling regeneration to take place. Here, we investigated a novel pNIPAM based hydrogel, which can be maintained as a liquid ex vivo and be injected into the IVD where the body temperature triggers in situ gelation. The hydrogel can be combined with extracellular matrix molecules (ECM), such as hyaluronic acid (HA), to produce a cellular scaffold that is sufficient enough by itself to induce targeted stem cell differentiation to the NP cell phenotype, without the need for external manipulation of cells or addition of growth factors.

Methods: Human MSCs were embedded into hydrogel systems and cell viability and migration characteristics assessed. The hydrogel scaffolds were characterized using scanning electron microscopy (SEM) to investigate morphology, pore characteristics and the potential influence of cells and deposition of matrix. Dynamic mechanical analysis (DMA) under a compression frequency scan was utilised to determine the mechanical properties of hydrogel constructs compared to native NP tissue. Differentiation capacity of the human MSCs was performed using qRT-PCR of NP ECM markers including: collagen type II (Col II) and aggrecan (Agg), as well as proposed NP markers cytokeratin-19 (Ker-19), laminin subunit alpha-5 (Lam-5) and ovostatin homolog-2 (OVO-2) (Minougue et al 2010, Rutges et al 2010). NP negative gene markers: collagen type I (Col I); runt related transcription factor 2 (Runx2); collagen X and integrin binding sialoprotein (IBSP) were also assessed. Matrix production was investigated using histological stains; Alcian blue for proteoglycans, Masson Trichrome for collagen and Alizarin red for calcium deposition and immunohistochemistry analysis of aggrecan and collagen type II.
**Results:** Viability of MSCs was maintained within hydrogel systems for the 6 weeks investigated, where the cells were shown to migrate through the hydrogel system, deposit matrix within and differentiate towards the NP cell phenotype. RT-PCR showed a switch from collagen type I to collagen type II, with expression of all ‘NP’ markers including Agg, Ker-19, Ovo-2 and Lam-5 observed whilst bone specific genes including Runx-2 and IBSP were down regulated. An initial increase in collagen type X was observed after 1 week, however this decreased again following 2 weeks. SEM analysis demonstrated hydrogels without cells contained pores approximately 10µm in diameter, whilst hydrogels containing cells showed matrix deposition, narrowing of pores and clear cells embedded within the matrix (Figure 1). Deposition of glycosaminoglycans (GAGs) was confirmed via positive Alcian blue staining and immunopositivity seen for aggrecan following 4 weeks (Figure 2). Collagen deposition was observed following 2 weeks as seen by increased Masson Trichrome staining together with immunopositivity for collagen type II (Figure 3). Alizarin red staining demonstrated no calcium deposition by the cells demonstrating no calcification of the matrix. DMA analysis demonstrated hydrogel systems containing 0.2% w/w hyaluronic acid displayed similar mechanical properties to native NP tissue.

**Discussion:** Here, we have developed a hydrogel system with the potential to deliver MSCs via minimally invasive injection using small bore needles which decreases the chance of inducing damage to the annulus fibrosus. We have demonstrated that the hydrogel systems were non-toxic, induced differentiation of MSCs without the need for additional growth factors and showed potential to provide mechanical support to the motion segment whilst regeneration takes place. The hydrogel combined with hyaluronic acid, presents a cellular scaffold where the structural environment alone is sufficient enough to induce differentiation towards the NP cell phenotype without the addition of chemical cues, thus simplifying the treatment strategy for the repair of IVD degeneration. Current investigations are focused on investigating this system within a hypoxic environment to assess whether NP cell differentiation of hMSCs in the hydrogel is accelerated or inhibited in conditions that mimic the degenerate IVD microenvironment.

**Significance:** Together this work will assess the potential of the hydrogel system as a novel injectable therapeutic for the repair and mechanical support of IVD degeneration a major cause of low back pain.
Fig 1: SEM images of A: pNIPAM:HA hydrogel (no cells); B,C,D: pNIPAM:HA + hMSCs following culture for 1 wk. Scale bar: 10μm (A), 30μm (B), 20μm (C) and 5μm (D).

Fig 2: Matrix production by hMSCs in pNIPAM:HA following culture for 4wks. A: GAG production stained with Alcian blue; B: Aggrecan immunohistochemistry.
Fig 3: Matrix production by hMSCs in pNIPAM:HA hydrogel following culture for 4wks: A: Collagen production stained with Masson Trichrome; B: Collagen type II immunohistochemistry.