AN INJECTABLE HYDROGEL FOR NUCLEUS PULPOSUS REGENERATION

A Thesis by

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The following faculty members have examined the final copy of this thesis for form and content, and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science, with a major in Biological Sciences.

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ABSTRACT

Intervertebral discs within the human spine act as shock absorbers between each of the vertebrae in the spinal column. As people age, disc cells are constantly subjugated to degenerating stress by various mechanical and environmental factors leading to musculoskeletal impairment and lower back pain. Nucleus pulposus cells (NP cells), jelly-like avascular tissue within the middle of the intervertebral disc, are crucial component of the disc Disc degeneration starts here in these cells. Research into regenerating the NP cells in degenerating intervertebral discs may provide a breakthrough in treating spine disorders. This project is designed to fabricate and characterize hydrogel composites to investigate the growth and viability of human nucleus pulposus (HNP) cells and the extracellular matrix gene expression by the HNP cells in the hydrogel. Specifically, we fabricated type II collagen and hyaluronic acid hydrogels that were cross-linked with the ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide crosslinker (NHS). The hydrogels were cross-linked using varying concentrations of the crosslinkers. HNP were seeded into crosslinked and non-crosslinked hydrogels. Results from cell viability assays such as live/dead assay and AlamarBlue assay showed cell growth and proliferation in both non-crosslinked and crosslinked hydrogels. Quantitative PCR assay demonstrated the extracellular matrix gene expression by the cells cultured in these gels. The results of gene expression studies indicated the formation of extracellular matrix by the cells and adaption of cells to the environment after long-term cell culture in these hydrogels. This study suggests that the type II collagen-HA hydrogel and crosslinked hydrogel with EDC at low concentration are a permissive matrix for the growth of HNP cells and can potentially be applied to NP repair.

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CHAPTER 1

INTRODUCTION

1.1 Degeneration of intervertebral discs

Lower back pain is a leading cause of disability which inflicts great physical encumbrance upon people and an equally distressing economic burden on the health care system. Lower back pain is the most common problem amongst individuals of age group 20-50 (1, 2). Management of health conditions through treatment and therapy, in monetary terms, accounts for about $50 – 90 billion in the US and £12 billion in UK (1, 3, 4). More importantly, lower back pain is the most common cause of musculoskeletal impairment, which has been reported to have a high impact on modern industrialized society. This and other effects make these spine related disorders one of the major maladies in medicine and impact not only daily lives but the country's economy as a whole.

Lower back pain is associated with degeneration of intervertebral discs (5). There are 24 intervertebral discs (IVD) in the human spine. The human spine is comprised of cervical vertebrae (C1-C7), followed by thoracic vertebrae (T1-T12), lumbar vertebrae (L1-L5) and sacral vertebrae at the end. IVD are the compound structures found between each vertebra and are linked with each vertebra through cartilaginous endplates (6, 7). IVD are 7-10 mm thick avascular structures comprised of three major regions: the thick outer ring annulus fibrosus (AF), the central NP and the cartilaginous end plate (6, 8) as shown in Figure 1. An IVD acts as a cushion to absorb shock and to support load transmission to the spine. It also provides flexibility and six degrees of multi axial spinal motion between each of the vertebrae in the spinal column. It can do this by keeping the vertebrae separated and by stabilizing forces within them when

there is an impact from any activity, mechanical loading and body weight. They also serve to protect the nerves that run down the middle of the spine and IVD (5, 7, 9).

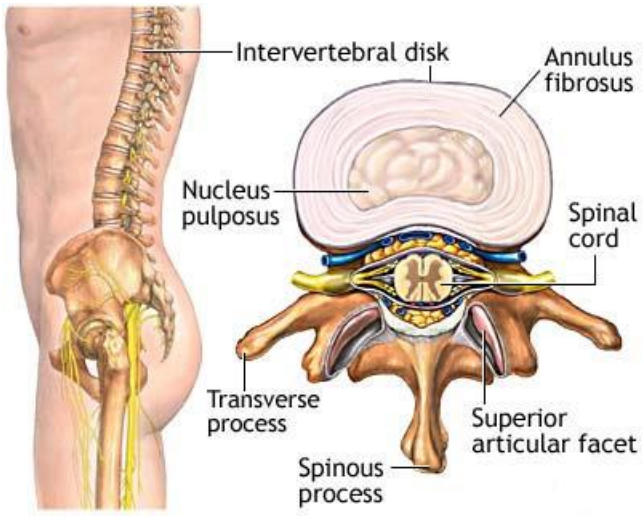


Figure 1: Schematic diagram showing an intervertebral disk. Figure adapted from http://www.physio-pedia.com/Thoracic_Disc_Syndrome

*Figure 2: Figure depicting stress exerted on the vertebral column due to various voluntary movement and external disruption.*Figure adapted from http://blog.corewalking.com/wp-content/uploads/2013/01/walking-and-lower-back-pain.jpg

1.1.1 Nucleus Pulposus (NP)

NP is a jelly-like avascular tissue in the middle of the intervertebral disc. NP is soft and gelatinous, sandwiched between the cartilage endplates. It functions to distribute hydraulic pressure in all directions within each disc under compressive loads. The extracellular matrix of the gelatinous NP is composed largely of water (70-90%), collagen type II, and aggrecan, a large aggregating hydrated proteoglycan (5, 10). NP cells are considered as the builder and maintainer of the extracellular matrix (10). The NP cells reside in an environment that has a limited vascular supply and generate energy through anaerobic glycolysis. Variation in NP cell morphology has been seen with age and maturation. In the case of humans, large and vacuolated cells with granular cytoplasmic inclusions similar to notochordal cells are seen at birth. However, after few years, cells become populated by smaller, round, and non-vacuolated cells, resembling articular cartilage (AC) chondrocytes which lead to the absence of notochordal cells in the embryonic tissue that then guide the formation of the spine and the nuclei pulposi (3, 5).

1.1.2 Annulus Fibrosis (AF)

AF is a tough and fibrous tissue that is composed of multiple overlapping layers and carries similarity to both ligament and large arteries (9). The AF of the intervertebral disc comprises of 15-25 concentric lamellae with the collagen fibers aligning parallel within each lamella and elastin fibers lying between the lamellae (8). Basically AF forms the inner region which is mostly comprised of type II collagen and fibrochondrocytes whereas the outer region consists of type I collagen and dense fibroblasts (6, 9). AF provides strength and crucial biomechanical support to the disc during mechanical loading and stressful environment conditions (7).

1.1.3 Cartilage end plate (CEP)

The cartilage endplate of the intervertebral disc is a horizontal layer made up of hyaline cartilage and interfaces. It is comprised of collagen fibers that run horizontal and parallel to the vertebral bodies (8). With direct connection with vasculature of the vertebral body. CEP serves as a capping for the IVD. The CEP has greater capillary density at the center than at the periphery (7).

1.1.4 Intervertebral matrix

The major components of the intervertebral matrix are collagen fiber and proteoglycan. Intervertebral disc exclusively consists of type I collagen at the extreme outer edge and type II collagen in the NP. Although type I and type II collagen constitute the major portion in AF and NP respectively, some other types of collagen such as type I, VI, IX, and XI are also found in the NP and type II, III, VI, IX, XI are found in the AF (6). Some significant functions of the collagen is to provide tensile strength to the disc, maintain a tough three dimensional network to support the cell and anchor tissues to the bone (8). Another major structural component of the matrix with substantial influence on the mechanical properties is the proteoglycans. Proteoglycans also influence organization of collagen fibers (6). One of the essential molecules of proteoglycan is the glycosaminoglycan side chains (GAGs) that act as water binding molecules and thus maintain the hydration in the NP (7). GAGs are the most abundant heteropolysaccharides in the body. Aggrecan is the dominant structural proteoglycan comprised of numerous monomers of non-covalently bound HA. Aggrecan is supposed to have essential involvement in chondrocyte-chondrocyte and chondrocyte- matrix interaction and provide viscoelastic structure to IVD (11).

The glycosaminoglycan keratin sulphate (KS) and chondroitin sulphate (CS) attach to aggrecan which in turn bind to HA via its N-terminal globular domain through interaction

facilitated by link protein. The negative charge associated with the proteoglycans trapped in the collagen matrix attracts cations and water in the matrix (Figure 3).

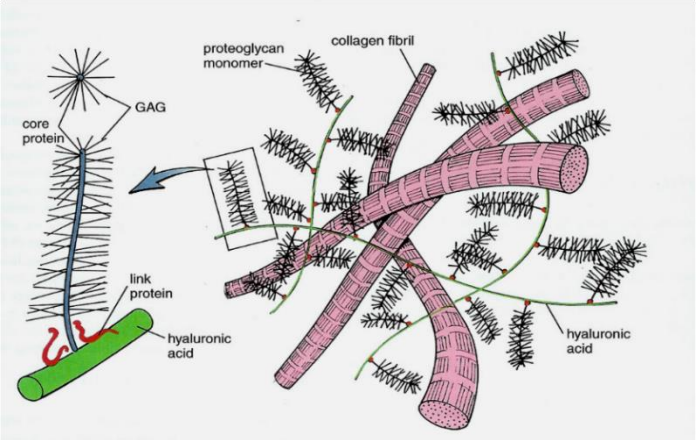


Figure 3: Figure showing the layout of intervertebral disk matrix inreraction

1.1.5 Degeneracy in IVD

Degradation in IVD with aging is a natural phenomenon (Figure 2). Although degradation in IVD cells occurs with increase in age, the environmental and genetic factors also contribute to the degradation process (6). Due to various voluntary movement and external disruption, disc undergoes complex combination of forces and deformation.(9). With aging, disc cells are constantly challenged by a stressful microenvironment such as hypoxia, a limited supply of nutrition, various mechanical loadings and environmental damages which leads to the alteration of ECM surrounding cells and neighboring cells (12, 13). In a normal healthy disc, NP is gelatinous, comprised of highly vacuolated chondrocytes as well as small chordoblasts; has major composition of type II collagen and proteoglycan and high water content that provide hydrostatic pressurization and resistance to compressive loading forces (9, 14). The normal NP is also avascular in nature and lacks nerve outgrowth which makes it immunologically privileged.

(14, 15). NP cells tend to decrease with age, lowering their density mainly because the cells themselves are not readily capable of self-repair (16).

During degeneration, NP is the site where specific characteristic tissue changes occur by dehydration leading to a decrease in NP size and increase in intradisc pressure. This results in increased stress on the AF with a compensatory increase in functional size. The hydrated gelatinous ECM is condensed and replaced by a more fibrous structure (12, 17). Over time the elastic properties of the NP begin to decrease, swelling properties become compromised; gradual weakening of the collagen filling, growth of nerve and vessel and cell death is seen. These alterations contribute to the reduced hydration properties and strength of NP leading to the inability to function and altered stress distribution to the disc (6, 9, 14).

In degenerated disc, disorganization in collagen and elastin network appears. The degenerated disc is characterized by the loss of proteoglycan and water from the disc which loosen up the nucleus and increases load bearing by the annulus. This leads to the bulging or rupture of AF. Also genetic susceptibility and matrix degrading enzymes are observed in the degenerated disc (18). The degenerated disc elicits an immune response generated by the proinflammatory signaling by disc cells. Fracture of vertebral body and localized mechanical disruption of CEP, AF and NP causes deregulation of interaction among them leading to the herniation in disc. When a herniated disc (Figure 4) presses on the surrounding nerve roots or dorsal root ganglion, it stimulates moderate to severe pain and numbness (14). Intervertebral disc degeneration (IDD) is characterized by an alteration at the cellular level, increased cell death, swelling of disc, gene polymorphism, immune privilege unbalance and aberrant gene expression (2, 19).Polymorphism in several genes such as vitamin D receptor gene, collagen IX alleles, metalloportese-3 and chondrion sulphate-1 domain of the aggrecan gene leads to IDD. Moreover polymorphism also inhibits the function of TGFβ and IL-1 which contribute to IDD.

Furthermore it has been reported that rate of IDD occurrence in a patient's relative is 75% higher than the non-related patients. (20, 21).

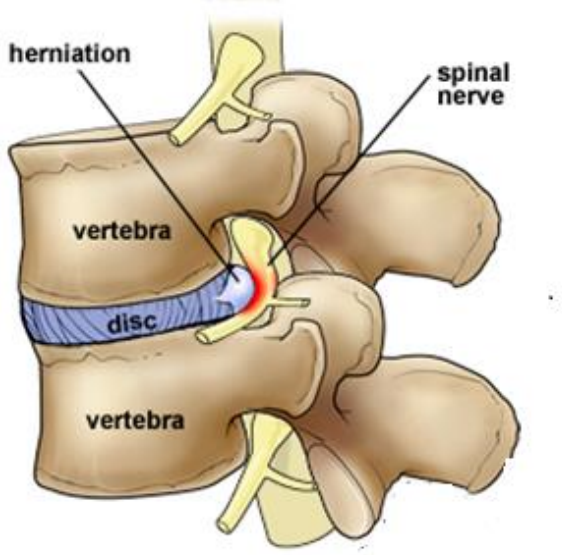


Figure 4:Schematic figure showing a herniated disc. Figure adapted from http://painrelief123.com/disc-decompression/

1.2 Approaches for disc regeneration and repair

Variable strategies have been investigated for regeneration and repair of intervertebral disc degeneration.

1.2.1 Biomaterials-based NP repair

The development of transplantable biomaterials provides a promising approach to regenerate IVD degeneration. Biomaterial scaffolds that can mimic the extracellular matrix of the native nucleus pulposus have been investigated for *in vitro* cell-biomaterial interaction (1, 4, 22-28) and *in vivo* transplantation into animal models with degenerative IVDs (29, 30) (Table 1). A few types of natural hydrogels such as collagen, hyaluronic acid (HA), fibrin, gelatin, alginate, and chitosan (1, 4, 22, 31-33) were investigated for their potential to replace and regenerate

nucleus pulposus. These injectable biomaterials have the ability to self-assemble to a higher-order network (34).

Natural biopolymers that mimic native ECM biochemistry and mechanical properties exhibited the ability to maintain cell viability and provide natural cues to cells that may stimulate a healing or regenerative response. Additionally, the natural materials allow the process of natural remodeling without the production of toxic byproducts (35-38). Type II collagen and HA, being major components of IVD, were studied for their ability to repair the NP. The biological and material properties of hydrogel generated by collagen and HA are normally enhanced by crosslinking. This optimal crosslinking method can stabilize the hydrogel, reduce the degradation rate, and control gel swelling with minimal toxicity to cells. In one study (4), 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) were used to crosslink the hydrogel composed of type II collagen and HA. Rat mesenchymal stem cells (MSCs) were grown in the hydrogels, and the cellular behavior in the gels was studied. Results showed that the hydrogel composite can support cell growth and proliferation. A quantitative polymerase chain reaction (PCR) showed increased expression of type II collagen for the cells in hydrogel without a significant increase of type I collagen, compared to the cell culture in a culture dish. In another study, hydrogel of type II collagen and HA were generated as an injectable hydrogel by crosslinking with poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) (1). The 4S-StarPEG improved the collagen/HA hydrogel's mechanical and degradation properties and supported bovine NP cell growth. However, it also decreased expression of type II collagen and aggrecan by the cells grown in the hydrogel after culturing for 14 days.

Hydrogel of other natural materials has also shown the potential for NP regeneration. In one study, lyophilized chitosan-gelatin scaffolds were prepared by crosslinking a chitosan and

gelatin solution. The growth of NP cells in the chitosan-gelatin scaffolds or alginate hydrogel was studied. After 21 days of cell culturing, cell counting showed a higher number of NP cells in the alginate hydrogel compared with that in the chitosan-gelatin scaffolds. This study suggested that the alginate scaffold generated a better condition for the proliferation of NP cells than the chitosan-gelatin scaffold (39). In a recent study, Mercuri et al. (40) generated a elastin-glycosaminoglycan-collagen (EGC) composite hydrogel with similar resilience and hydrophilic properties of the NP. This material showed the ability to restore its original dimensions and water content after multi-cycle mechanical compression and the ability to resist the enzymatic degradation. Human-derived adipose tissue stromal cells (hADSCs) were cultured in hydrogel for 14 days. The expression of type II collagen and aggrecan increased with minimal expression of type I collagen. The hydrogel was transplanted into subdermal pockets of the dorsal mid-line of male juvenile Spargue Dawley rats. This *in vivo* study showed that the hydrogel was biocompatible and resistant to enzymatic degradation. Synthetic materials have also been investigated for NP repair (29, 30, 41). Poly(ethylene glycol) (PEG) hydrogels have been widely used in tissue-engineering applications because of their non-toxic and hydrophilic properties (42). To facilitate NP cell-matrix interactions and cell-mediated matrix remodeling, a PEG hydrogel with tunable mechanical properties was functionalized with ECM ligand-laminin. The gel-carrying NP cells were injected into a rat tail IVD. After 14 days, the NP cell retention in cultured IVD explants was significantly higher when cells were delivered by a PEG-LM111 hydrogel than the cells in liquid suspension (41).

Peptide materials mimicking the nanoscale of a natural ECM are synthetic and biologically easily modifiable. The biocompatible peptide nanofiber scaffolds have a high density of bioactive epitope and are biodegradable in the body without immunogenicity. In an experiment performed by Wang et al. (26), a link N nanofiber scaffold (LN-NS) was self-

assembled by mixing a peptide solution of RLN (AcN–RADARADARADARADAGG DHLSDNYTLDHDRAIH–COHN2) and RADA16 (AcN–RADARADARADARADA–COHN2). Rabbit NP cells were seeded on the surface of hydrogel. LN-NS scaffold was not toxic to the cells. The scaffold promoted NP cell adhesion, and stimulated cell migration and biosynthesis compared to that of the pure RADA 16 scaffold. Gene expression analysis suggests that there is an increment in the proteoglycan and type II collagen expression for the cells in the LN-NS scaffold than the pure RADA16 scaffold. This study suggested that the self-assembled peptide nanofiber scaffolds are advantageous in NP repair.

The nonangiogenic or antiangiogenic feature is an important aspect to be considered in the design of a hydrogel for nucleus pulposus regeneration. In one study, a chorioallantoic membrane (CAM) assay was performed to test the angiogenic response of gellan gum (GG)-based hydrogels. The ionic-crosslinked methacrylated GG (iGG-MA), and photo-crosslinked methacrylated GG (phGGMA) hydrogel discs were implanted in the CAM at day 10 of embryonic development. Results revealed that iGG-MA and phGG-MA hydrogels were not cytotoxic and did not permit the ingrowth of endothelial cells and therefore restricted vascular invasion (24). This study suggested that GG-based hydrogels functioned as a physical barrier for vascular invasion and are promising NP substitutes in the treatment of a degenerated IVD.

A combined construct with both external annulus structure and inner hydrogel center can mimic the entire IVD and provide an *in vitro* approach to study NP regeneration. A biphasic biomaterial structure with silk protein for the AF and fibrin-hyaluronic acid gels for the NP were constructed to mimic the entire IVD (43). The porcine AF cells were seeded on the toroidal scaffold that was formed of lamellar and porous silk, and the porcine chondrocytes were encapsulated in a fibrin-HA hydrogel in the center of biphasic construct. The lamellar scaffolds supported the AF-like tissue over two weeks, and the porcine chondrocytes demonstrated the NP

phenotype within the hydrogel. After culturing for four weeks, the gene expression of type I collagen and aggrecan at the AF-NP region and type II collagen at the NP region increased significantly.

Table 1: Biomaterials for NP regeneration

Biomaterials	**Cell type**	**Model**	**Approach**	**Outcome**	**Ref**
Alginate, chitosan-gelatin scaffold	Human NP cells	*In vitro*	NP cells were grown in hydrogel.	Cell count was higher in alginate scaffold than that in chitosan-alginate scaffold.	(39)
EGC	Human ADSCs	*In vitro* / *in vivo* (rat)	hADSCs cells were grown in EGCs. In *in vivo* study, EGC hydrogel was placed into subdermal pocket.	Hydrogel exhibited shape-memory sponge characteristic and supported stem cell viability and differentiation towards NP cell-like phenotype.	(40)
PEG	Pig NP cells	*In vitro*	Laminin functionalized PEG hydrogel containing NP cells was injected into IVD explant.	NP cell retention in cultured IVD explants was significantly higher over 14 days for cells in PEG-laminin hydrogel compared to cells in liquid suspension.	(41)
Peptide nanofiber	Rabbit NP cells	*In vitro*	Cells were seeded on surface of scaffold.	Scaffolds promoted NP cell adhesion, migration, and biosynthesis.	(26)
Silk, fibrin and HA	Human chondrocyte	*In vitro*	Chondrocyte cells were cultured in hydrogel with 10% serum.	Cells in fibrin/HA hydrogel with 1.5% silk showed superior expression of type II collagen, SOX-9, and aggrecan.	(44)
Type II collagen and HA	Calf NP cells	*In vitro*	Cells were grown in hydrogel cross-linked with PEG.	Gel was not toxic to cells and supported cell growth.	(1)
Type II collagen and HA	Rat MSCs	*In vitro*	Cells were injected into hydrogels.	Hydrogel cross-linked with EDC (8mM) presented optimal swellability and cell proliferation rate.	(4)

Table 1 (continued)

C/Gp gel	Human MSCs	*In vitro*	Cells were grown in C/Gp gel.	MSCs grown in C/Gp hydrogel showed differentiation of MSCs to a phenotype similar to both articular chondrocytes and NP cells.	(45)
Nanofibrous scaffold enveloping a HA hydrogel center	Human MSCs	*In vitro*	Cells were seeded in biophasic scaffold.	Time-dependent development of chondrocytic phenotype of seeded cells.	(27)
Small intestine submucosa scaffolds	Human AF and NP cells	*In vitro*	Cells were seeded into scaffold.	Cell metabolism was improved. Gene expression of collagens I, II, and X, aggrecan, and Sox-9 was maintained.	(28)
PGA- hyaluronan		*In vivo* rabbit	Constructed hydrogel scaffold was inserted into defected disc of rabbit.	After 12 months of implantation, histological studies showed cell migration into the defect and formation of repaired tissue.	(29)
PLGA	Beagle dog NP cells	*In vivo* beagle dog	PLGA scaffold with NP cells were implanted into disc.	Survival and proliferation of NP cells in IVDs of beagle model were observed after 8 weeks.	(30)

1.2.2 Cell therapy in IVD regeneration

NP cells are of mesenchymal origin (37, 46) and MSCs can differentiate into tissues of mesenchymal origin. MSCs that are extracted from the trabecular bone(46-48) , cartilage endplate(49), umbilical cord blood (50), synovial tissue (51, 52), and adipose tissue (53, 54), have been investigated for NP regeneration (Table 2). Stem cell therapy is an alternative source of NP cells for IVD regeneration. Stem cell-mediated cell therapy can potentially restore the function and structure of degenerated disc (35, 55). Though no specific marker has been identified for NP cells, they resemble that of chondrocytes. In recent years, MSCs have been utilized in therapies for a few animal models for IVD regeneration (47, 48, 50, 54, 56, 57). The application of MSCs retrieved the disc height and increased the population of the extracellular matrix, thus improving spine function in these studies.

The co-culture of MSCs with NP cells significantly upregulated the biological activity of NP cells. In a study, a co-culture of human NP cells isolated from the lumbar disc and bone marrow MSCs resulted in enhanced cell proliferation, DNA synthesis, and proteoglycan synthesis by the NP cells. Neither chromosome abnormalities nor tumorigenesis in the human NP cells were observed in this study (58). In another study, a co-culture of human synovial MSCs with rat NP cells significantly changed the gene expression profile of the NP cells. The expression of the genes for degradation enzymes and inflammatory cytokines (52) was inhibited. This *in vivo* study demonstrated that MSCs assumed a similar role as NP cells in the transplanted IVD and can effectively restore its structure. In a study, either MSCs or NPs were seeded in a degenerated rabbit disc for IVD regeneration. Sixteen weeks after cell transplantation, the decline of the disc height was reduced and the T2-weighted signal intensity increased for groups with the MSCs or NP cell transplant compared with the non-transplanted control animals. A gene

expression assay revealed a significant up-regulation of type II collagen and aggrecan gene expression in the groups with MSCs or NP cell transplantation, compared with the non-transplanted group (47). Another *in vivo* study (46) was performed to transplant the MSCs into a degenerated IVD of the beagle dogs. Results showed that the MSC transplantation induced disc regeneration. The NP region with the transplanted MSCs expressed the Fas-ligand (FasL) protein, suggesting that MSCs are likely responsible for conservation of the IVD immune privilege.

A recent study reported that the cartilage endplate-derived stem cells (CESCs) showed a greater potency of chondrogenesis and osteogenesis than the bone marrow MSCs (49). CESCs isolated from the cartilage in the end plate of a degenerated human IVD can differentiate into cells of multilineage. Although the CESCs showed similar morphology as bone marrow-derived MSCs, they had significant higher chondrogenesis and osteogenesis ability compared with the bone marrow MSCs. Annulus fibrosus-derived stem cells (AFSCs), NP cells, CESCs, and bone marrow MSCs were implanted into rabbit IVDs, and the results of IVD regeneration were compared. Magnetic resonance imaging (MRI) and histological samples revealed the strongest NP regenerative effect in the animals with CESC transplantation compared with other cells. This study suggested that CESCs may serve as an efficient cell source for NP regeneration.

The implantation of poly (L-lactic-co-glycolic acid) (PLGA) into an IVD was performed in the animal model. In one study, PLGA scaffolds were transplanted into the defected IVD of the rabbits and the implant promoted IVD tissue repair. Cell migration and proliferation were also observed in the defective tissue. Further studies tried the potency of biomaterials for stem cell delivery to treat degenerated IVDs (30). Biomaterials can simultaneously provide structural support for IVDs and serve as a carrier for stem cell delivery.

The advantage of biomaterials for cell delivery is that scaffolding produces a permissive microenvironment to facilitate cell survival and growth. It was reported that alginate gels delivering different types of stem cells were injected into the rabbit IVDs for regeneration. The alginate gel with CESCs showed the strongest NP regenerative effect compared with AFSCs, nucleus pulposus-derived stem cells (NPSCs), and MSCs (49). Clinically, collagen sponges carrying bone marrow MSCs were transplanted into degenerated IVDs of two patients. Two years after surgery, symptoms of lumbago, leg pain, and numbness in both patients were alleviated. T2-weighted magnetic resonance imaging showed that the signal intensity of the intervertebral discs with cell grafts was high, suggesting high moisture content. Roentgenkymography confirmed the improvement of instability of the lumbar disc. The outcome of this therapy is encouraging, indicating that the combination of biomaterials and stem cells is an optimal approach for IVD regeneration (38).

Table 2: Stem cell transplantation in NP regeneration

Cell type	Source of cells	Model	Approach	Outcome	Ref
NPCs, MSCs	Human NP cells and human bone marrow MSCs	*In vitro*	NP cells and MSCs were co-cultured.	NP cell proliferation was enhanced, as well as DNA synthesis and PG synthesis.	(58)
NPCs, SDSCs	Synovial tissue of pig knees	*In vitro*	NP cells and SDSCs were grown in decellurazied extracellular matrix.	SDSCs showed enhanced viability and guided SDSCs differentiation toward NP lineage.	(51)
hUCB-MSCs	Human neonatal umbilical cord blood	*In vitro*	hUCB-MSCs were injected into rabbit IVD explant.	hUCB-MSCs showed chondrocyte like lineage.	(50)
MSCs, NPCs	Rabbit MSCs from femoral bone and rabbit NP cells	*In vivo* rabbit	NPCs or MSCs were injected into NP of rabbit IVD.	Sixteen weeks after cell transplantation, in MSC- or NPC-transplanted groups, a decline in the disc height index was reduced. T2-weighted signal intensity increased compared with the sham-operated group.	(47)
MSCs	Canine iliac crest	*In vivo* canine	MSCs were injected into discs at different concentrations as 10^5, 10^6, and 10^7cells per disc.	Transplantation of 10^6 MSCs showed the best effect on maintenance of IVD structure and inhibition of IVD degeneration compared to cell numbers of 10^5 or 10^7.	(48)

Table 2 (continued)

MSCs	Beagle dog iliac crests	*In vivo* dog	MSCs were transplanted into degenerated IVD.	Regeneration of IVD was observed. Differentiated MSCs expressed FasL.	(46)
Synovial MSCs	Rabbit knee joint	*In vivo* rabbit	Cells were transplanted into degenerated IVDs.	Disc structure was maintained by enhanced production of type II collagen. 2. Expression of degradative enzymes and inflammatory cytokines was inhibited.	(52)
ADSCs	Human lipoaspirated fat tissue	*In vivo* rabbit	ADSCs were injected into injured rabbit lumbar IVD.	ADSC-injected discs showed improved extracellular matrix production and little ossification of damaged cartilage in nucleus pulposus compared with degenerative control discs.	(54)
CESCs, FSCs, NPSCs, MSCs	Human IVD	*In vivo* rabbit	Alginate gels containing different type of cells were injected into IVDs.	CESCs showed strongest NP regenerative capacity compared with other cell types.	(49)
MSCs	Human illium	*In vivo* human	Collagen sponge delivering MSCs were grafted into degenerative IVD.	MRI imaging showed that signal intensity of intervertebral discs with cell grafts was high, thus indicating high moisture content 2 years after surgery.	(38)

1.2.3 Molecular therapy in NP repair

Growth factors can maintain IVD homeostasis by regulating phenotype and functions of IVD tissue, controlling cell proliferation and differentiation, and promoting synthesis of the ECM (59, 60). However, the short half-life time of growth factors limits their therapeutic application. Gene therapy may generate long-lasting biological effect by introducing exogenous genes into the targeting cells and subsequently produce desired therapeutic product to treat diseases (61). In gene therapy, the desired gene can be inserted into the cells by non-viral or viral vector (62). The delivery of the desired gene into the target cell can be achieved by direct *in vivo* gene transduction or *ex vivo* gene transduction followed with *in vivo* transplantation of the transduced cells (20).

MSCs and NP cells are ideal for genetic modification and they can serve as gene carriers in the therapy. *In vitro* studies of gene transduction to the cells exhibited the enhanced ability of extracellular matrix generation that is necessary for tissue modification after the cells are introduced with particular functional genes(23, 33, 43, 63, 64) (Table 3). In a study by Bucher et al. (63), MSCs that were transfected with growth and differentiation factor 5 (GDF_5) were cultured as a monolayer or in algnate. Transfected MSCs in the 3D culture showed greater up-regulation of aggrecan, chondrogenesis marker SOX 9 and discogenesis marker keratin type I cytoskeletal 19 (KRT19) compared to the 2D culture. This study suggested that the GDF_5 is a potential candidate molecule for gene therapy in IVD regeneration. In another experiment (64), human NP cells were transduced by the lentiviral vector-delivering human telomerase reverse transcriptase (hTERT) gene and the expression of hTERT in human NP cells as detected up to 210 days post-viral infection. The restoration of telomerase activity and delayed cell senescence was observed. Also, the expression level of collagen II and aggrecan increased.

Direct injection of gene vectors into the IVD generated a significant therapeutic effect (22, 32, 34, 65, 66), which relies on the transduction of the virus to the endogenous cells. The adeno-associated virus serotype 2 (AAV2) vector-delivering anabolic bone morphogenetic protein 2 (BMP2) or anticatabolic tissue inhibitor of melloproteinase 1 (TIMP1) was injected into the punctured discs of Zealand white rabbits (66). MRI imaging, and histological and biochemical testing showed alleviation of disc degeneration for the viral vector-treated group compared to the untreated group. The transplantation of gene-modified cells into the IVD may enhance ECM generation and remodel the degenerated tissue. In one study, NP cells expressing human bone morphogenetic protein 7 (hBMP7) were injected into cryopreserved IVDs and the constructed IVDs were transplanted into beagle dogs (65). After 24 weeks of transplantation, the beagle dogs transplanted with the IVDs injected with NP cells expressing h-BMP_7 showed minor degeneration compared to the IVD allografts with non-hBMP7-expressing NP cells or without NP cells. This study also demonstrated that NP cells expressing hBMP7 could survive at least 24 weeks and prevents the degeneration of the transplanted IVD.

Although gene therapy seems promising for the treatment of IVD degeneration, some hurdles cannot be neglected. The recombinant viral vectors have been intensively investigated for cell transduction and show promising results for the therapy of degenerative NP. However, the immunogenicity, toxicity, and the concern of insertional mutagenesis limit its application clinically. Though non-viral gene vectors provide a safer alternative approach, the transfection efficiency is normally low. Additionally, long-term effects of the gene vectors for NP regeneration have not been established. While considering the monetary aspects of gene vectors and growth factors, gene therapy is not a cost-effective solution.

Table 3: Gene therapy in NP regeneration

Genes	Delivery system	Cell type	Model	Approach	Outcome	Ref
L-hTERT	Lentivirus	Human NP cells	*In vitro*	Cultured human NP cells were transduced with lentiviral vector.	Telomerase activity and delayed cell senescence were restored. Collagen II and aggrecan increased.	(64)
GDF_5	Electroporation	Human MSCs	*In vitro*	Transfected cells were cultured in alginate. Transfected cells were injected into IVD papain degeneration organ culture system.	Aggrecan and SOX 9 for cells in alginate gel were up-regulated. GAG/DNA ratio for IVD papain degeneration organ culture system with cells was partially recovered.	(63)
TIMP-1, BMP-2	Adenovirus	Human NP cells	*In vitro*	Cultured NP cells were transduced with virus encoding TIMP-1 or Ad-BMP-2.	Synthesis of proteoglycan increased.	(67)
BMPs, Sox9,	Adenovirus	Bovine NP cells and AF cells	*In vitro*	Cultured NP and AF cells were transduced with virus encoding rhBMP-7 or Sox9.	Transduced NP and AF cells expressed appropriate gene to maintain their phenotype.	(68)
IL-1Ra	Adenovirus	Human NP cells and AF cells	*In vitro*	Cultured NP and AF cells in monolayer or in alginate were transduced with adenoviral vector encoding IL-1Ra gene.	Increased levels of IL-1Ra inhibited production of IL-1 and prevented degeneration of IVD.	(69)
h-BMP_7	AAV2	Dog NP cells	*In vivo* dog	IVDs of beagle dogs were injected with NPCs expressing h-BMP_7.	IVDs injected with NPCs expressing h-BMP7 showed minor degeneration.	(65)

Table 3 (continued)

lacZ	Adenovirus	Rabbit NP cells	*In vitro/ in vivo* rabbit	Cultured NP cells were transduced with Ad-lacZ. Ad- lacZ was injected into rabbit lumber IVD.	In vitro and in vivo studies demonstrated nucleus pulposus cells were efficiently transduced by Ad-lacZ.	(70)
BMP2, TIMP1	AAV2		*In vivo* rabbit	Discs were punctured and treated with AAV2 vector encoding of either BMP2 or TIMP1.	Treated discs showed less degeneration than untreated discs.	(66)
OP1,SOX9	AAV		*In vivo* rabbit	Mixture of AAV-OPI and rAAv-SOX9 were injected into rabbit IVDs.	Combined effect of OP1 and SOX9 significantly increased disc height and expression of disc proteoglygan and typeII collagen.	(71)
TGF	Adenovirus		*In vivo* rabbit	Adenovirual vector encoding hTGFβ1 was injected into rabbit lumbar disc.	Increased proteoglycan synthesis indicated enhanced biological activity of intervertebral disc.	(72)

1.3 Hydrogel

Hydrogel refers to a chemically or physically formed network of polymers having highly absorbent property. They possess a significant degree of flexibility comparable to that of the natural tissue due their high water content. Biodegradable hydrogel has been used as a very attractive component for encapsulating cells and provides them with native conduit environment for regeneration, growth and differentiation (73). Hydrogels are ideal materials for implantation because they introduce low levels of foreign matter into the body and allow high diffusivity of biomolecules(74). Many hydrogels mixed with cells have been tested for IVD repair (75, 76). Over the past decade, a variety of naturally- and synthetically-derived materials have been utilized to form injectable hydrogels for tissue engineering applications due to their excellent biocompatibility (77). Some of these types of hydrogels are listed in Table 4.

Table 4: Table showing a list of different natural and synthetic hydrogel

	Natural	**Synthetic**
Hydrogel	Collagen	poly(ethylene glycol)
	Hyaluronic acid	
	Alginate	
	Fibrinogen	
	Chitosan	
	Agar/Agarose	

1.3.1 Collagen Hydrogel

Collagen, a major component of the ECM, has potential biomedical applications, provides cellular adhesion, can be enzymatically modified and in some cases is required for the proper differentiation of specific cell types (78-80). There are varieties of collagen and type I collagen is most frequently used to form hydrogels for different cell studies (75) and few

attempts have been made to use type II collagen for cell transplantation. Several strategies have been developed to improve the chemical properties and mechanical rigidity of collagen to provide better environment for cell growth and differentiation.

Type II collagen twists together and arranges into long, thin fibrils and forms cross-links to one another in the spaces around cell matrix. It is specific for cartilaginous tissues and is essential for the normal embryonic development of the skeleton, for linear growth and for the ability of NP cells to resist compressive forces.

Other types of collagen such as type I, II, III, VI, IX and XI are found in the extracellular matrix.

1.3.2 Hyaluronic Acid

HA is the major component of the extracellular matrix (ECM). The backbone of HA is composed of disacharidD-glucuronic acid and N-acetyl-D glucosamine repeats (81, 82) . Its function is to bind water and lubricate movable parts of the body such as joints and muscles. The biological functions of HA between its fragmented form (low-molecular weight polymer or oligosaccharides) and the native polymer form (high molecular weight polymer) are different (83). Low molecular weight HA stimulates the expression of inflammatory genes by a variety of immune cells through binding to cell surface proteins and high molecular weight HA is generally a bio-inert molecule that acts to maintain a hydrated and porous environment, absorbs mechanical shock and regulates osmotic balance and can also sequester and gradually release growth factors and other bioactive molecules to communicate a local biological influence over cell (82, 83). The structural and biological properties of HA mediate its activity in cellular signaling, wound repair, morphogenesis, and matrix organization which can be modified mechanically and chemically for number of biomedical applications(84).

1.3.3 Peptide hydrogel

Self–assembling (SAP) peptide has attracted much attention from researchers as the major molecular scaffold material of the biological world at the nano-scale, micro-scale, and macro-scale and also for advantages such as flexibility in molecular design, improved biocompatibility and the ease of materials preparation(85, 86).

1.3.4 Synthetic hydrogel

Recently, synthetic hydrogel is receiving attention to use as a biomaterial for 3D cell culture. PEG-based polymers are widely used for 3D cell studies, because of their flexibility for chemical modifications with various functional groups including cross-linking groups, bioactive epitopes, stimulus responsive units, and biodegradable units and also influence the grafted cells within the graft (75, 87).

1.4 Crosslinker

Crosslinkers are the homobifunctional or heterobifunction reagents that establish either inter-molecular or intra-molecular interaction by forming covalent bonds between two proteins. The covalent interaction involves end groups that react with functional groups such as primary amines, sulfhydryls, carbonyls, carbohydrate and carboxylic acids that can be utilized to determine the tertiary structures of protein, geometric arrangements of subunit within a protein or complex and to study protein- protein or ligand-receptor interactions (88). Different crosslinkers are commercially available with varying lengths of spacer arm or bridges (the structure that join two reactive groups together). It is best to determine the correct arm length for the establishment of the interactions between the two reactive groups. Basically the crosslinker with short spacer arm is used for the intermolecular interactions whereas the crosslinker with long spacer arm is used to establish intra-molecular interaction (89).

Crosslinker are being applied to provide various hydrogels with stiffness and desired mechanical properties like load bearing capacity and remolding process for tissue engineering applications (90). Crosslinkers such as PEG (1), gold nanoparticles (91), dendrimer (92), EDC (4, 93) etc are being utilized for various tissue engineering purposes to enhance the mechanical properties of the hydrogel scaffold. EDC (Figure 5), a water-soluble zero-length crosslinker, can crosslink the molecules directly and, EDC is less toxic than bridge-linking crosslinkers. So in this study, type II collagen and HA is crosslinked with EDC. The EDC can form amide bond for the amine and carboxyl groups Figure 6.

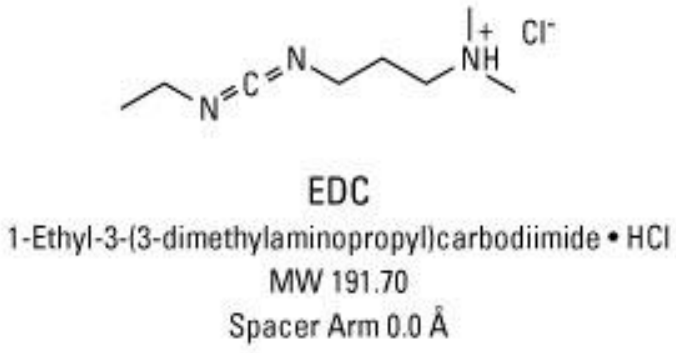


Figure 5: Figure showing an EDC molecule. Figure adapted from https://www.lifetechnologies.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/carbodiimide-crosslinker-chemistry.html

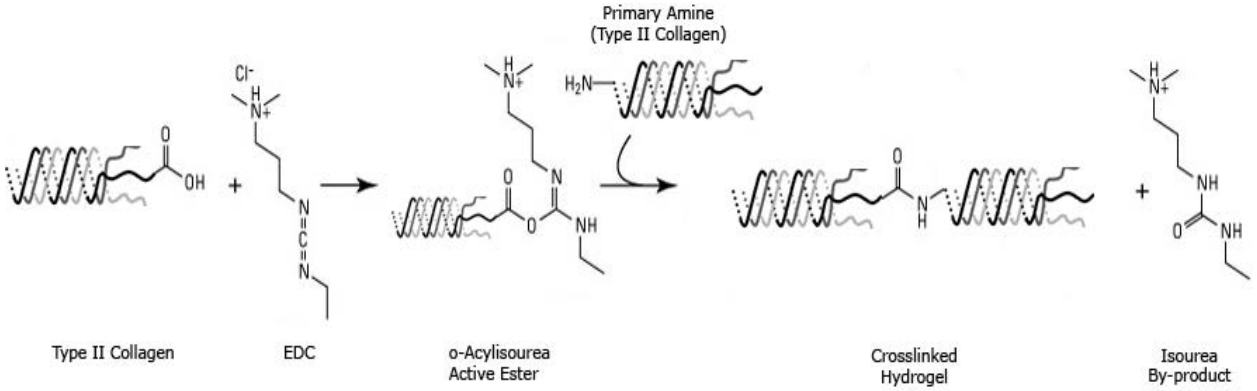


Figure 6: EDC mediated reaction chemistry.

CHAPTER 2

RESEARCH OVERVIEW

2.1 Introduction

The intervertebral disc (IVD), an elastic structure between adjacent vertebrae of the spine, is mainly composed of two types of fibrocartilage materials: the external annulus fibrosus (AF) and the inner nucleus pulposus (NP). Type II collagen, aggrecan and the aggregating hydrated proteoglycan make up the extracellular matrix (ECM) of the gelatinous NP (5, 10). This extracellular matrix helps in absorbing the hydraulic pressure within each IVD thus providing the capability to cope with compressive loads to the NP. Thus, NP cells are considered as the builder and maintainer of the extracellular matrix (10). Degeneration of the IVD is characterized by specific tissue changes in the NP cells mainly caused by dehydration leading to a decrease in NP size followed by a decrease in intradisc pressure. This directly hampers the ability of the NP cells to absorb and distribute the hydraulic pressure between IVDs causing exertion of more stress on the AF which then results in various painful symptoms and diseases such as lower back pain, neck pain and other spinal disorders. Moreover, NP cells tend to decrease with age, lowering their density mainly because the cells themselves are not readily capable of self-repair (16). With the process of degeneration and ageing, NP cell density constantly decreases and lost the ability to proliferate and generate ECM (10, 94).

Researchers have been focused on various approaches to regenerate the lost NP cells and rejuvenate the IVD function in order to alleviate the painful symptoms and diseases associated with IVD degeneration that are both a physical and an economic burden. One such approach involves the use of biomaterial scaffolds and has long been a topic of interest (1, 4, 29, 39, 44,

95). A number of studies focus on the use of synthetic and natural hydrogels in order to replace and regenerate NP cells using collagen, hyaluronic acid (HA), fibrin, gelatin, polyglycolic acid, alginate and chitosan (1, 4, 39, 44, 95). This approach is most effective since the NP cells themselves have only limited ability of self-repair (16, 31, 96). The study reports a successful prevention of disc degeneration by transplanting rabbit NP cells into animal IVD (47). Another study showed that use of type II collagen-HA hydrogel can support the growth of rat mesenchymal stem cells or bovine nucleus pulposus cells (1, 4). A study showed that collagen neural conduits crosslinked with 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide (EDC) can considerably sustain peripheral nerve regeneration (97). Collagen microspheres crosslinked with EDC can support oligodendrocyte precursor growth for myelination *in vitro* (98). .

The biomaterials that are more suitable for grafting into the NP are type II collagen and HA since these biomaterials can be easily remodeled by endogenous or transplanted cells without production of byproducts. However, successful use of these biomaterials as a therapeutic alternative to IVD-related ailments is still far from achievable. With an aim to enhance the use of these biomaterials for NP cell regeneration and repair, we conducted a thorough analysis of the use of type II collagen and HA with a crosslinked and non crosslinked matrix for replenishing perishing NP cells in the IVD. In this study, we performed an *in vitro* study by the fabrication of an injectable type II collagen-HA hydrogel crosslinked with a low amount of EDC. By growing human nucleus pulposus cells in this hydrogel, we investigate the growth and gene expression of these cells. Our study indicates that type II collagen-HA hydrogel crosslinked with low concentration of EDC is an effective and suitable medium for the growth of human NP cells and also serve as cell carriers for human NP cell transplantation into the IVD.

2.2 Summary of the project

Nucleus pulposus (NP) cells are considered the most crucial component for the intervertebral disc function. Regeneration and growth of NP cells in degenerating intervertebral disc is currently a field of concern. This study is particularly based on application of tissue engineering strategies to generate biomaterial scaffolds (hydrogel) that provide a permissive environment for NP cell growth for forming new tissue. It has been hypothesized that type II collagen and hyaluronic acid hydrogel that are crosslinked with EDC can support human nucleus pulposus cell growth and proliferation and maintain the extracellular matrix gene expression. This study aims to develop an injectable, biodegradable, and biocompatible hydrogel comprised of collagen type II and hyaluronic acid (HA) and investigate the biological behavior of human NP cells in the hydrogel. The research had been designed to fabricate the hydrogel and characterize the properties of the hydrogel, evaluate the viability of cell seeded in the hydrogel and analyze the gene expression of the extracellular matrix. The hydrogels, when strengthened by crosslinking with ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), were found to decrease the gel degradation rate caused by collagenase and reduce the uptake of phosphate-buffered saline (PBS) solution. Human nucleus pulposus (HNP) cells were found to proliferate in both the crosslinked and non-crosslinked hydrogels by performing an AlamarBlue assay. These cells showed multiple processes and proliferation in the EDC-crosslinked (0.1 mM) type II collagen-HA hydrogel after the cells were cultured for 2 months. Compared to cells cultured for 8 days, a culture after 30 days showed a significant decrease in the gene expression of type II collagen of the cells in crosslinked gel (0.1mM EDC) and of the aggrecan of the cells in the non-crosslinked gel. On the other hand, the gene expression of type I collagen in both the crosslinked (0.1mM EDC) and non-crosslinked gel

increased with the increase in culture time from 8 to 30 days. Collectively, this study indicates that the crosslinked type II collagen and hyaluronic acid hydrogel (0.1mM EDC) is a more preferable and suitable hydrogel composite for HNP cells growth and proliferation.

2.3 Hypothesis

Type II collagen and hyaluronic acid hydrogel that are crosslinked with EDC can support human nucleus pulposus cell growth and proliferation and maintain the extracellular matrix gene expression.

2.4 Specific aims of the project:

Aim 1: Fabrication and characterization of corsslinked type II collagen and hyaluronic acid hydrogel:

The optimized protocol has been generated and various tests have been done for the fabrication and characterization of hydrogel.

1. Standardization of the hydrogel fabrication protocol
2. Fourier-transformed infrared (FTIR) spectroscopy test. Investigation of chemical bond formation between hyaluronic acid and type II collagen of EDC/NHS cross-linked hydrogels.
3. Swelling test – The swelling test for different crosslinked and non-crosslinked hydrogels were performed by the measurement of relative PBS uptake.
4. Degradation test- To study the comparative degradation properties of different crosslinked hydrogels with respect to non-crosslinked hydrogel.

Aim 2: Investigation of the growth and viability of human nucleus pulposus cells in the hydrogel.

1. Live/dead viability assay: To examine the viability of cells those are grown in the hydrogel.
2. Alamar blue viability assay: To measure cell proliferation in the hydrogel.
3. Cell morphology study by scanning electron microscopy: To determine the structure and form of cells seeded in hydrogel.

Aim 3: Investigation of the gene expression of extracellular matrix by human nucleus pulposus cells in the hydrogel.

Real time RT-PCR study will be performed to determine the gene expression of extracellular matrix i.e aggrecan, type I collagen and type II collagen by the NP cells in the hydrogel.

CHAPTER 3

MATERIALS AND METHODS

3.1 Fabrication of type II collagen-HA hydrogels

The source of type II collagen used in this study is fetal bovine cartilage. The collagen solution was lyophilized at -80°C and kept at -20^{o}C for application. It was dissolved in 0.01 M acetic acid to make a final concentration of 9mg/ml, and stored at 4^{o}C with gentle rocking. Hyaluronic acid (Sigma-Aldrich, St. Louis, MO) was dissolved in Dulbecco's modified eagles medium (DMEM) (Lifetechnology, Grand Island, NY) along with 0.4 M NaCl at 1.57 mg/ml. Type II collagen (9 mg/ml) and a HA-sodium solution were mixed together with a ratio of 9:1 by weight. The final solution was then adjusted to a pH of 7 using 1 M NaOH aqueous solution and 5X phosphate-buffered saline (PBS) solution. After adjusting the pH, the hydrogel was crosslinked with different concentrations of EDC (Sigma-Aldrich, St. Louis, MO) (0.1, 0.5, and 1 mM). A control hydrogel without any crosslinker was also prepared.

3.2 Infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) was performed to study the chemical bond formation between the HA and the type II collagen using EDC. Spectra were obtained with a Spectrum 100 FT-IR Spectrometer Perkin Elmer (PerkinElmer, Waltham, MA). To obtain an IR spectrum, the specimen (hydrogel) was loaded onto a salt plate which was then placed on a mounting plate in the sample-loading compartment. The parameters were adjusted as per the protocol. In FTIR, the infrared beam enters the compartment, passes through the sample, and finally enters the detector area, resulting in a spectrum with peaks. The obtained peaks were then analyzed for results.

3.3 Swelling test

For the swelling test, crosslinked type II collagen-HA hydrogel specimens and non-crosslinked control samples were prepared, and the weight of each specimen was measured. The hydrogels were soaked in PBS solution and incubated for 2 hours at 37°C. Then the PBS solution was removed, and the hydrogels were held over a paper filter until all PBS had dripped off. The samples were then observed and weighed again. The weights obtained for each type of crosslinked and non-crosslinked hydrogels after soaking in the PBS solution were compared.

3.4 Degradation test

Non-crosslinked hydrogels and hydrogel specimens crosslinked with 0.1, 0.5 or 1 mM EDC were freeze-dried for 2 days, after which the weight of each sample was measured. Then the samples were digested with collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO), which was prepared in 0.1 mM Tris (pH 7.4) containing 0.05 M calcium chloride ($CaCl_2$). The samples were incubated in the digestion solution at 37°C for two different time durations: 5 hours and 11 hours. After respective time periods of incubation, the samples were centrifuged, the supernatant was removed, and the pellets were lyophilized. The weight of each sample was measured again. Mass loss for each time point was calculated by analyzing the initial mass (W_i) with the final mass (W_f) according to the following equation:

$$Mass\ loss(\%) = \frac{Wi - Wf}{Wi} \times 100$$

3.5 HNP cell culture

During discectomy surgery, intervertebral disc tissues from discogenic low back pain patients were obtained for this study. The Human Subjects Committee (HSC) of University of

Kansas Medical School –Wichita examined and exempted all experimental procedures. The nucleus pulposus (NP) portion of the tissue was minced to 1mm segments and digested with 0.25% trypsin for 30 minutes followed by addition of 0.2% collagenase for 4 hours with agitation. After filtration through a 100um Nylon Mesh (Fisher Scientific, Cat no. 22363549) to eliminate the tissue debris, the isolated cells were pelleted by centrifugation at 500g for 10 minutes. The cells were grown in a DMEM/ nutrient mixture F12 (Ham) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Lifetechnology, Grand Island, NY). The medium was changed after every 2–3 days. Cells were counted using hemocytometer and equally distributed, re-suspended in new flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2.

3.6 Cell growth in type II collagen-HA hydrogel

The type II collagen (0.5 ml, 9 mg/ml) and HA solution were added to the wells of a 48-well plate. The pH was adjusted to 7 using NaOH solution. A crosslinker (EDC) was added to the gel in different concentrations and mixed thoroughly a using pipette. Hydrogel without any addition of cross-linker was also prepared as the control. The HNP cells were seeded into both the crosslinked and non-crosslinked collagen-HA solution and incubated for 15 minutes at 37°C to allow the gel formation. Finally, the medium was added into each cell culture well. The medium was changed every 3 days. A microscopic view of the seeded cells in the medium is shown in Figure 7.

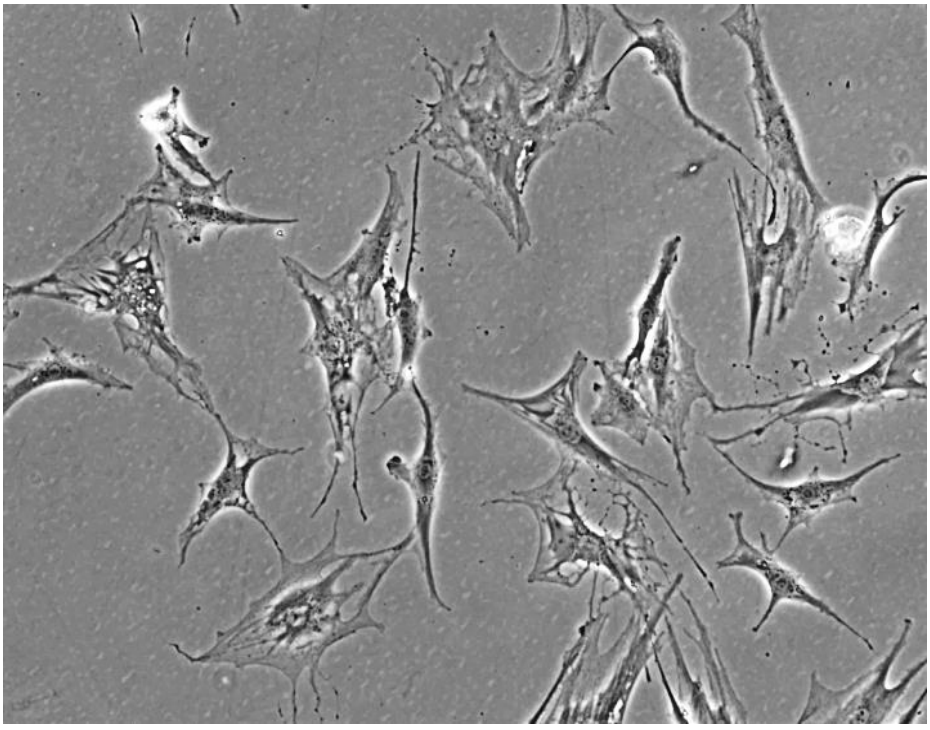


Figure 7: Microscopic view of HNP cells in Type II Collagen-HA hydrogel

3.7 Cell viability assay

The cell viability assay (LIVE/DEAD® Cell Vitality Assay, Life technology, Grand Island, NY) was performed under two time points: after 10 days and after 2 months of cell culture in the hydrogel at 37°C in a humidified atmosphere of 5% CO_2. The reagents for the LIVE/DEAD assay were ethidium homodimer-1 (Ethd-1), with a molecular weight of 856.77, and Calcein AM, with a molecular weight 994.87. These reagents were also purchased from Life technology. The LIVE/DEAD reagent stock solutions were removed from the freezer and allowed to warm to room temperature. EthD-1 stock solution (2 µl, 2 mM) and Calcein AM stock solution (0.5 µl of 4 mM) were added to sterile PBS solution (1 ml) and vortexed. The solution (300 µl) was added directly to each cell culture well and incubated for 30 minutes at room temperature. The cells were then viewed under a fluorescent microscope.

3.8 AlamarBlue assay

The viability and proliferation of HNP cells in the hydrogel were studied by monitoring their metabolic activity using the AlamarBlue assay (Pierce Biotechnology, Rockford, lL). This assay was also conducted under two time points: after 10 days or after 2 months. To perform this assay, HNP cells with a density of 50,000 cells were seeded in the hydrogel and cultured for respective time periods. These cells were then incubated with a cell culture medium containing 10% (v/v) AlamarBlue reagent for 4 hours. AlamarBlue reagent, 10% of the volume of cell culture medium, was added and autoclaved for 15 minutes of liquid autoclave cycle for the positive control. Negative control was prepared with AlamarBlue reagent, 10% of the volume of cell culture medium, without autoclaving. Absorbance was measured at wavelengths of 570 nm and 600 nm in a microplate reader (Synergy Mx Monochromator-Based Multi-Mode Microplate Reader, Winooski, VT).

3.9 Electron scanning microscopy

Cells cultured in the collagen-HA hydrogel were fixed in 2% glutaraldehyde-PBS solution for 30 minutes. The samples were broken into small pieces, dehydrated with graded ethanol, and dried with hexamethyldisilazane. Then they were air-dried and subsequently coated with gold. The images of the scaffolds with cells were taken by scanning electron microscopy (SEM) using a ZEISS SIGMA VP (Carl Zeiss Microscopy, LLC, Thornwood, NY).

3.10 Real-time PCR

For performing the quantitative reverse transcription polymerase chain reaction (qRT-PCR), 120,000 cells were seeded in each collagen-HA hydrogel. The total RNA of the HNP cells that were cultured for either 8 days or 30 days was extracted using an RNeasy Micro Kit (Qiagen, Germantown, MD) as per the supplier's protocol. The amount of RNA was determined

using a NanoDrop2000C (Thermo Scientific, Waltham, MA). The cDNA was reverse-transcribed from the total RNA using a High-Capacity cDNA Reverse Transcription Kit (Lifetechnology, Grand Island, NY) according to the manufacturer's protocol. The qRT-PCR was performed using the Power SYBR® Master Mix by the StepOnePlus™ qRT-PCR System (Lifetechnology, Grand Island, NY) at 95°C for 10 minutes, and then 40 cycles at 95°C for 15 seconds followed by 60°C for 60 seconds. Gene transcription was normalized in relation to transcription of the housekeeping rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression for each target gene. Primers used in the qRT-PCR are provided in Table 5.

Table 5: Primers used for qRT-PCR

Gene	Oligonucleotide (5'-3')
Type II collagen A1 (COL2A1)	F: CCGGGCAGAGGGCAATAGCAGGTT
	R: CAATGATGGGGAGGCGTGAG
Aggrecan	F: CCAGTGCACAGAGGGGTTTG
	R: TCCGAGGGTGCCGTGAG
Sox9	F: CATGAGCGAGGTGCACTCC
	R: TCGCTTCAGGTCAGCCTTG
Type I collagen A1 (COLIA1)	F: CGATGGCTGCACGAGTCACAC
	R: CAGGTTGGGATGGAGGGAGTTTAC
GAPDH	F: CGAGATCCCTCCAAAATCAA
	R: TTCACACCCATGACGAACAT

3.11 Statistics

Statistical analysis was done using the two-tailed Student's t-test, where data are expressed as the mean ± standard deviation. Statistical significance was placed at $p < 0.05$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Generation of crosslinked type II collagen and HA hydrogel

We have generated crosslinked type II collagen and HA hydrogel in the ratio of 9:1 by the adding 0.1mM, 0.5mM and 1mM concentration of EDC crosslinker, and the gel were stable in the cell culture medium. When the gel was transferred in injection needle and re transferred in glass tube no deformation in gel was observed as shown in Figure 8. The result showed the formation of the hydrogels with different concentration of EDC.

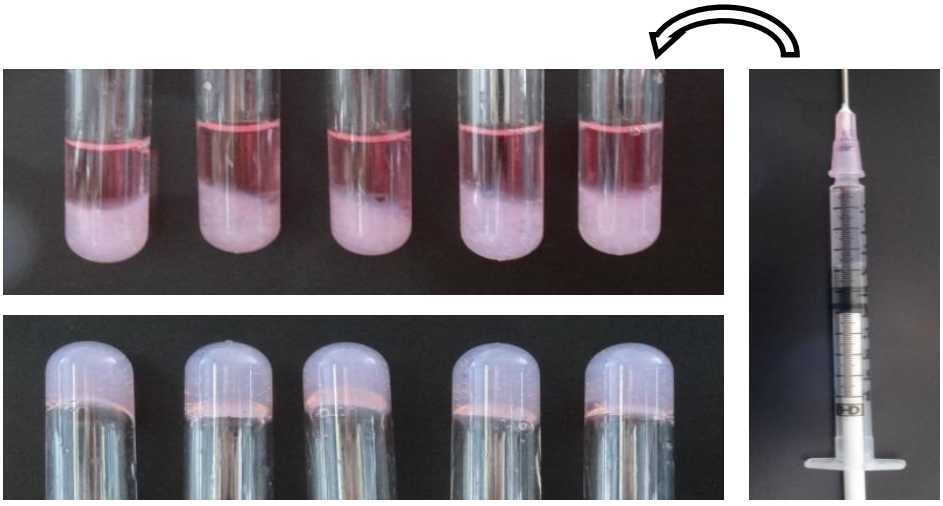


Figure 8: Type II collagen-HA hydrogels formed in glass tubes with cell culture medium. The hydrogel was found to remain stable at bottom of glass tubes after the removal of medium

4.1.2 Infrared Spectroscopy showing the amide group formation in the crosslinked hydrogel

Fourier transformed infrared spectroscopy was employed to determine the functional group of type II collagen and hyaluronic acid. Figure 9 shows the spectra obtained from FT-IR for the different cross-linked and non-crosslinked type II collagen and HA hydrogels. The formation of amide bonds was observed when the hydrogel was crosslinked with EDC. Result from FTIR showed the distinct bond formation for crosslinked hydrogels.

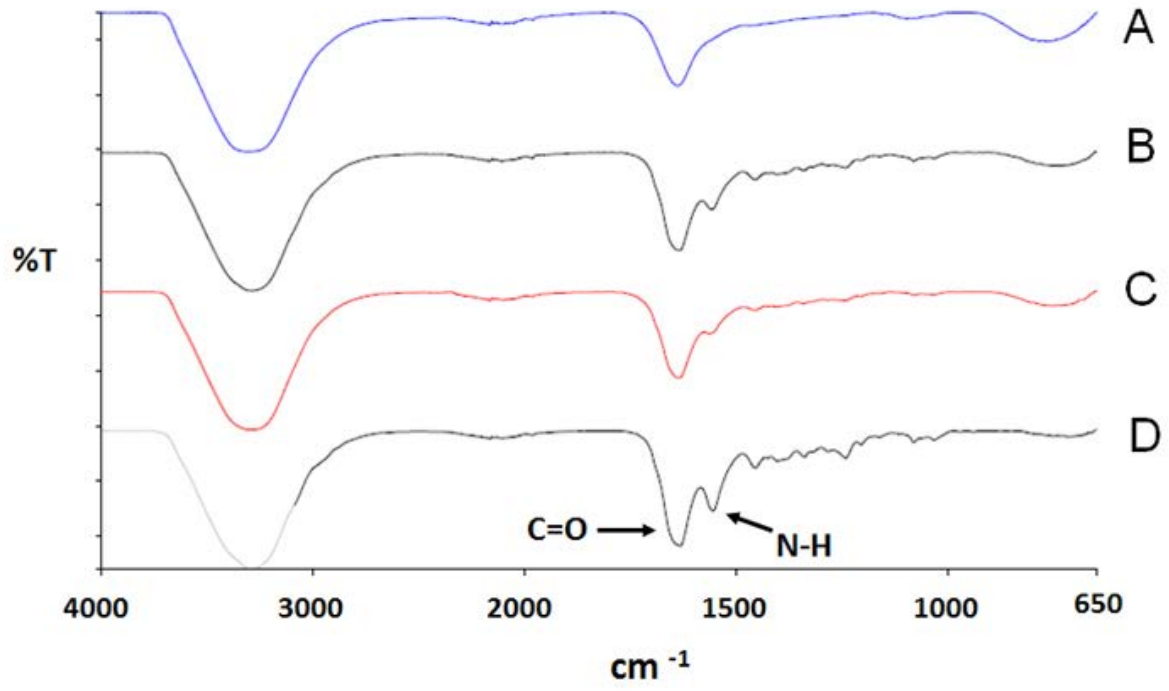
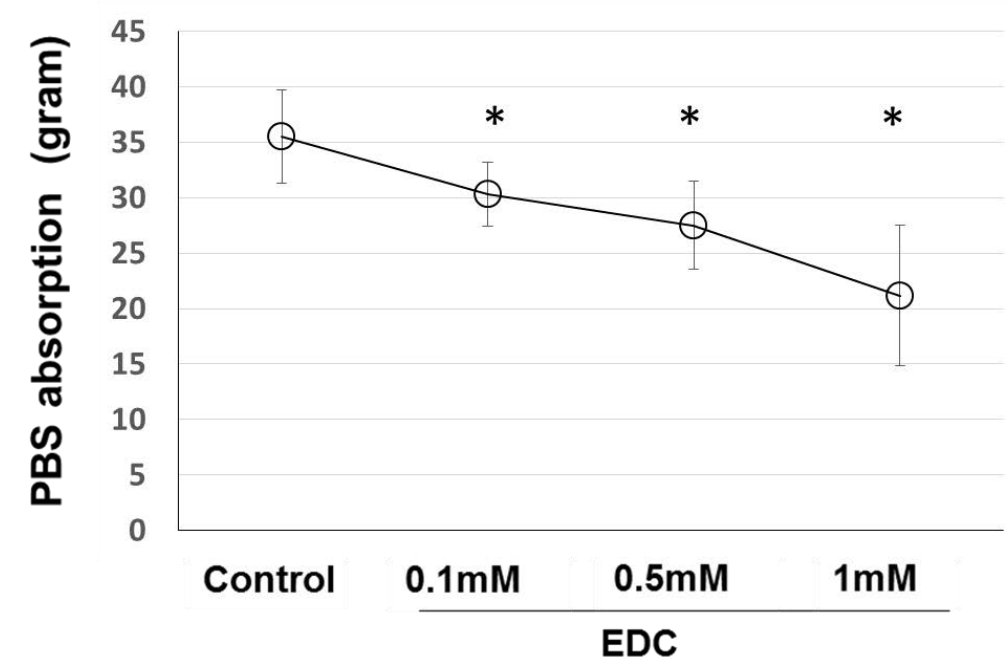


Figure 9: FTIR spectra of type II collagen/HA hydrogels: (A) non-crosslinked hydrogel; (B) gel crosslinked with 0.1 mM EDC; (C) gel crosslinked with 0.5 mM EDC; (D) gel crosslinked with 1mM EDC.

4.1.3 Swelling Test

The swelling test for different cross-linked and non-cross-linked hydrogels was assessed by the measure of relative PBS uptake. The PBS solution uptake for the non-crosslinked gel was 35.5 ± 4.23 mg. The PBS solution uptakes of the hydrogels crosslinked with 0.1 mM EDC, 0.5 mM EDC and 1 mM EDC were 30.33 ± 2.86 mg, 27.5 ± 3.94 mg, and 21.16 ± 6.33 mg, respectively. The results indicate that EDC crosslinking significantly reduced the uptake of PBS by the hydrogel. Figure 10 shows a plot of the PBS uptake in the different hydrogels. Results indicate that EDC crosslinking significantly reduced uptake of the PBS solution by the hydrogel.

A



B

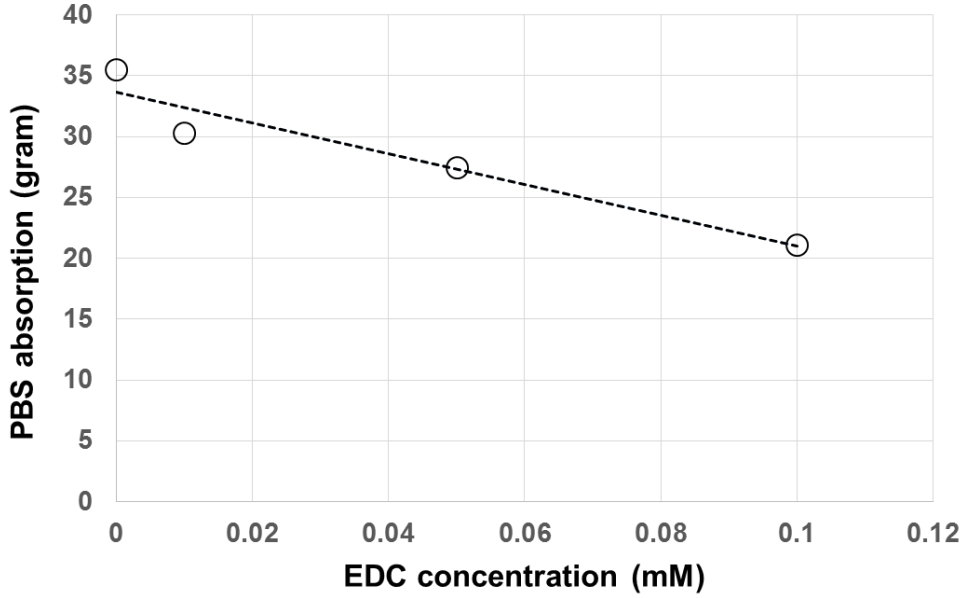


*Figure 10: (A) Relative PBS solution uptake of type II collagen-HA hydrogels compared with non-crosslinked hydrogel and hydrogels crosslinked with EDC at different concentrations. *, $p < 0.05$, compared with the non-crosslinked gel. (B) The correlation analysis of PBS absorption and EDC concentration. $R^2 = 0.9282$, $p=0.036$.*

4.1.4 Degradation Test

The increase of EDC concentration for crosslinking the type II collagen-HA hydrogel increased the resistance of the gels to degradation by collagenase. As shown in Figure 11, after incubation with collagenase for 5 hours and 11 hours, the degradation rate of the freeze-dried scaffolds decreased from 45.4 ± 1.4% and 99.2 ± 1.2% to 14.6 ± 11.9% and 56.1 ± 21.3%, respectively, when the EDC amount was increased from 0 mM to 1 mM.

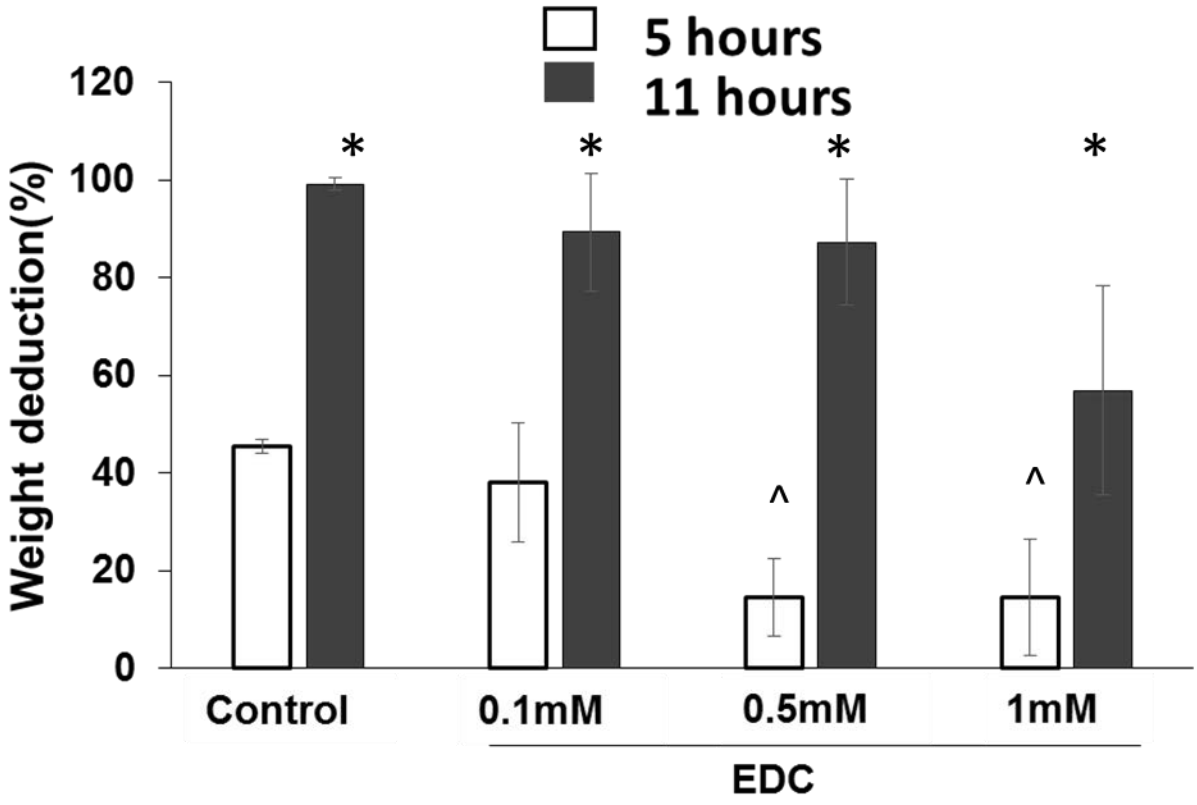


*Figure 11: Weight deduction of the type II collagen- HA hydrogel after 5 hours and 11 hours of collagenase treatment. *, $p < 0.05$, compared with the corresponding gels of 10 days. ^, $p < 0.05$, compared with the non-crosslinked gel.*

4.1.5 Live/dead viability assay

Cell viability was determined by using a live dead assay reagent kit and viewed under a fluorescent microscope (Figure 12 and Figure 13). After 10 days of cell culture in hydrogel, most cells cultured in control gel (95.21 ± 1.33 %) and in hydrogel cross linked with 0.1mM EDC/NHS (90.92 ± 3.90%) were found to be alive while the cells cultured in 0.5mM EDC/NHS crosslinked hydrogel were 76.03 ± 16.34% alive and 65.69 ± 8.78% of the cells were alive in 1 mM EDC/ NHS crosslinked hydrogel. After 2 months of cell culture, the live cells in the control gel and hydrogel crosslinked with 0.1 mM EDC were 93.86 ± 1.50% and 93.72 ± 0.39% respectively, while the live cells in the hydrogel crosslinked with 0.5 mM EDC and 1mM EDC respectively were 83.92 ± 8.02% and 50.90 ± 11.60%, respectively. Cells cultured in the control gel (Figure 14A) and gel crosslinked with 0.1 mM EDC (Figure 14C) after 10 days showed elongation. Cells cultured in the control hydrogel (Figure 14B) and the hydrogel crosslinked with 0.1 mM EDC/NHS (Figure 14D) after 2 months showed growth, elongation and proliferation. Live/dead assay indicate survival and morphology of cells seeded in hydrogel.

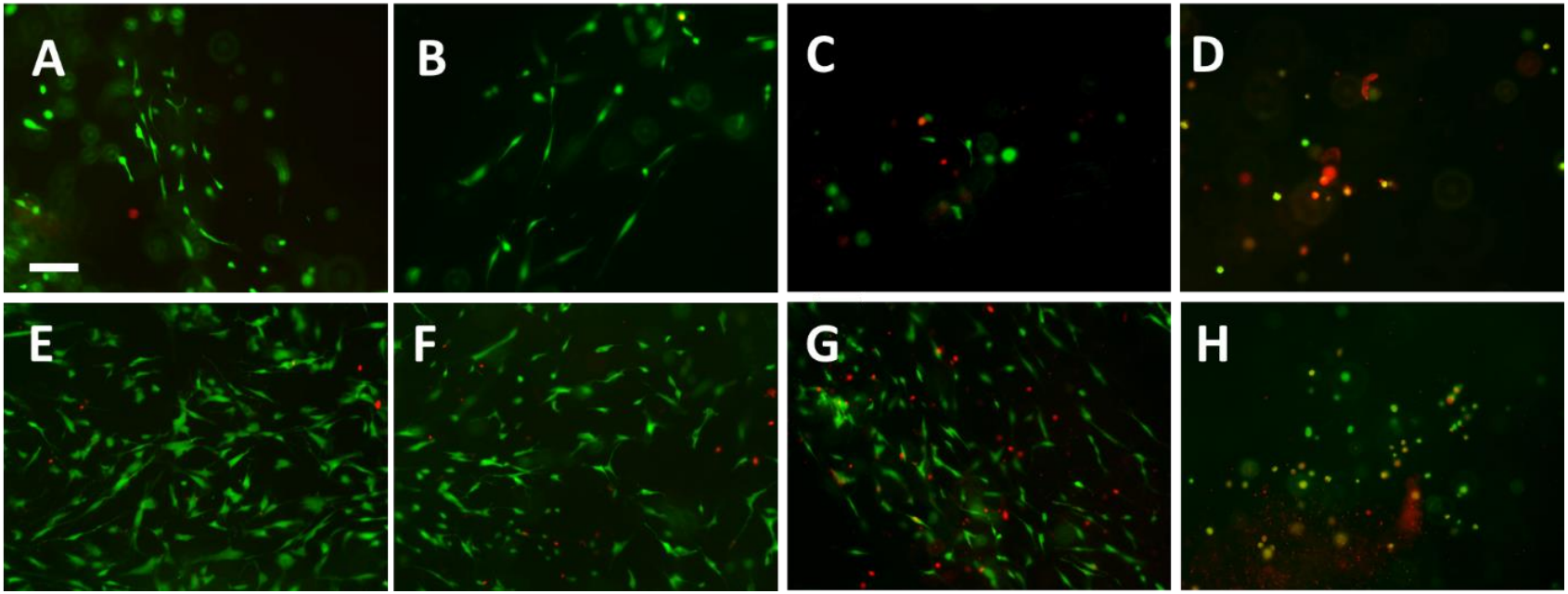


Figure 12: LIVE/DEAD cell vitality assay for HNP cells grown for 10 days in gel: (A)–(D) LIVE/DEAD cell vitality assay for HNP cells grown in gel for 10 days. (A) non-crosslinked hydrogel, (B) hydrogel crosslinked with 0.1 mM EDC, (C) hydrogel crosslinked with 0.5 mM EDC, and (D) hydrogel crosslinked with 1 mM EDC. (E) – (H)LIVE/DEAD cell vitality assay for HNP cells grown for 2 months in gel: (E) non-crosslinked hydrogel, (F) hydrogel crosslinked with 0.1 mM EDC, (G) hydrogel crosslinked with 0.5 mM EDC and (H) hydrogel crosslinked with 1 mM EDC. Live cells labeled with calcein AM (green). Dead cells labeled with ethidium homodimer-1 (red). Scale bar: 100 μm.

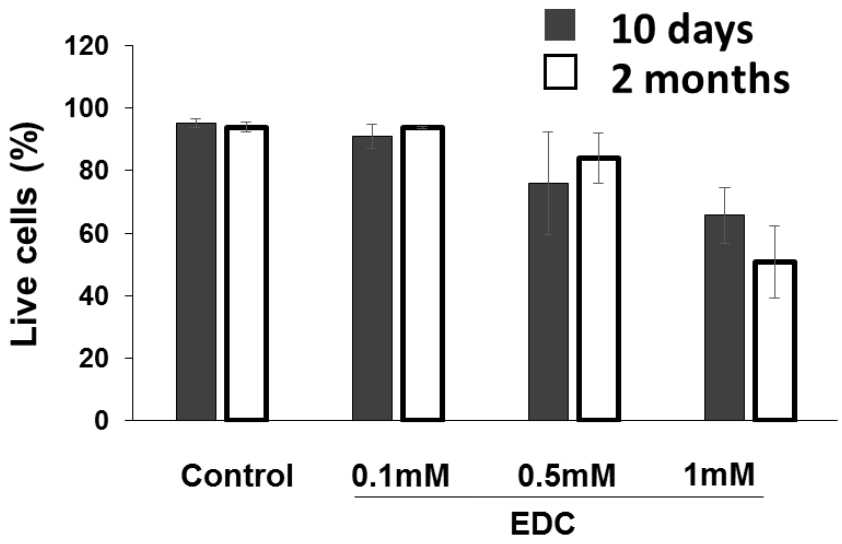


Figure 13: Graph showing pecent of live cells in hydrogel with no amd varying levels of crosslinking with EDC after 10 days and two months

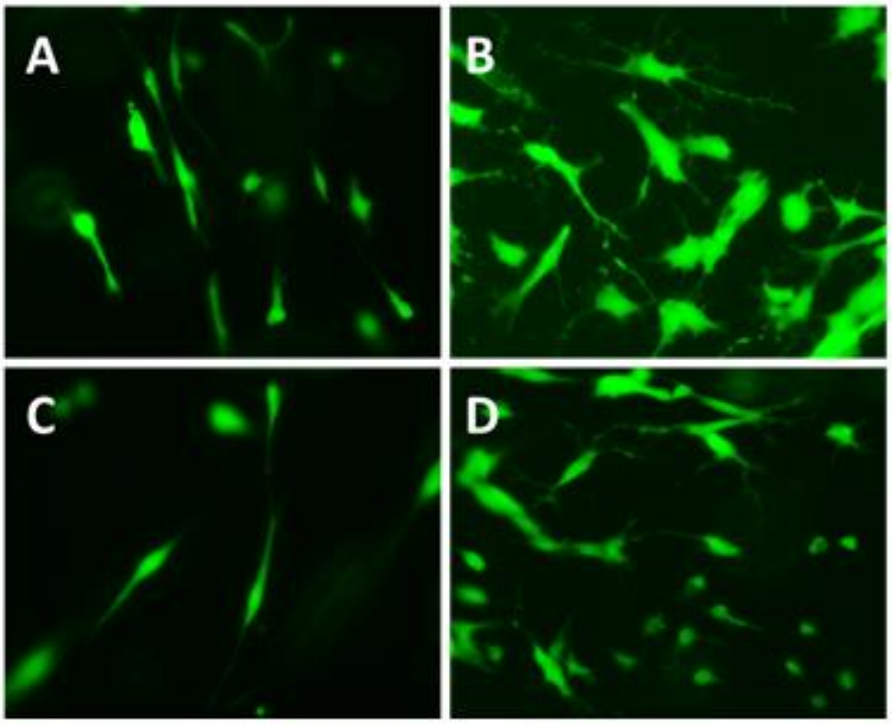


Figure 14: Morphology of HNP cells in hydrogel: (A) HNP cells in non-crosslinked type II collagen-HA hydrogel for 10 days; (B) HNP cells in non-crosslinked type II collagen-HA hydrogel for 2 months; (C) HNP cells in crosslinked type II collagen-HA hydrogel (0.1 mM) for 10 days; (D) HNP cells in crosslinked type II collagen-HA hydrogel (0.1 mM) for 2 months. Cells labeled with calcein AM. Scale bar: 50 µm

4.1.6 AlamarBlue Assay

Reduction of alamarblue reagent indicated cell proliferation in both crosslinked and non-crosslinked hydrogel. HNP cells cultured for 2 months showed significant increment in reduction compared to the cells cultured for 10 days. The reduction of AlamarBlue reagent by HNP cells in the control gel was 22.43 ± 3.11%, in 0.1 mM EDC was 17.5 ± 1.76% and 1 mM EDC was 14.37 ± 0.79% and was significantly increased at 30 days culture to 34.28 ± 6.05% for control, 32.31 ± 0.67% for 0.1 mM EDC, 8.44 ± 4.488% for 0.5 mM EDC and 24.483 ± 0.74% for 1 mM EDC. This is shown in detail in Figure 15. The AlamarBlue assay showed that HNP cells proliferated in both the control collagen-HA gel and the crosslinked collagen-HA gels.

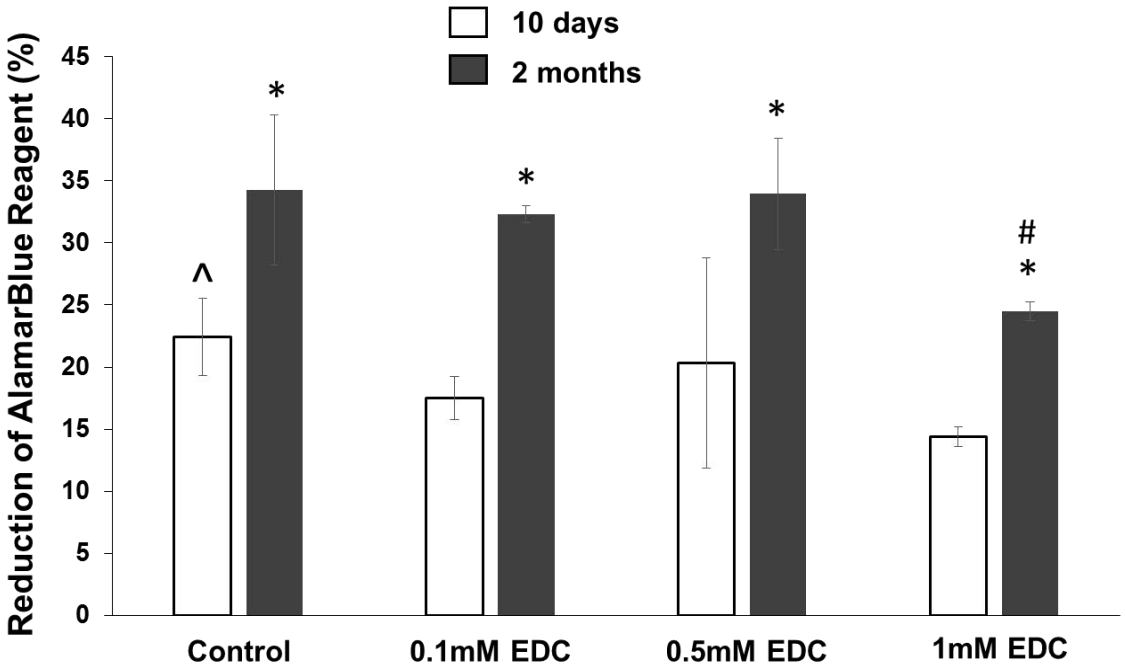


*Figure 15: AlamarBlue cell viability assay of HNPs growing in non-crosslinked and crosslinked collagen type II-HA hydrogel. *, $p < 0.05$, compared with the corresponding gels of 10 days. ^, $p < 0.05$, compared with the gels crosslinked with 0.1mM and 1mM EDC. #, $p < 0.05$, compared with the control gel and the gels crosslinked with 0.1mM and 0.5mM EDC.*

4.1.7 Scanning Electron Microscope (SEM)

1. The cells cultured in 0.1mM EDC/NHS crosslinked hydrogel for 2 months were viewed under SEM to determine the cell morphology by ZEISS SIGMA VP (Carl Zeiss Microscopy, LLC, Thornwood, NY). Figure 16 shows the SEM image reflecting multiple processes of the cells cultured in hydrogel. SEM imagines indicates the morphology of cells seeded in hydrogels. The cells developed multiple processes in the gel.

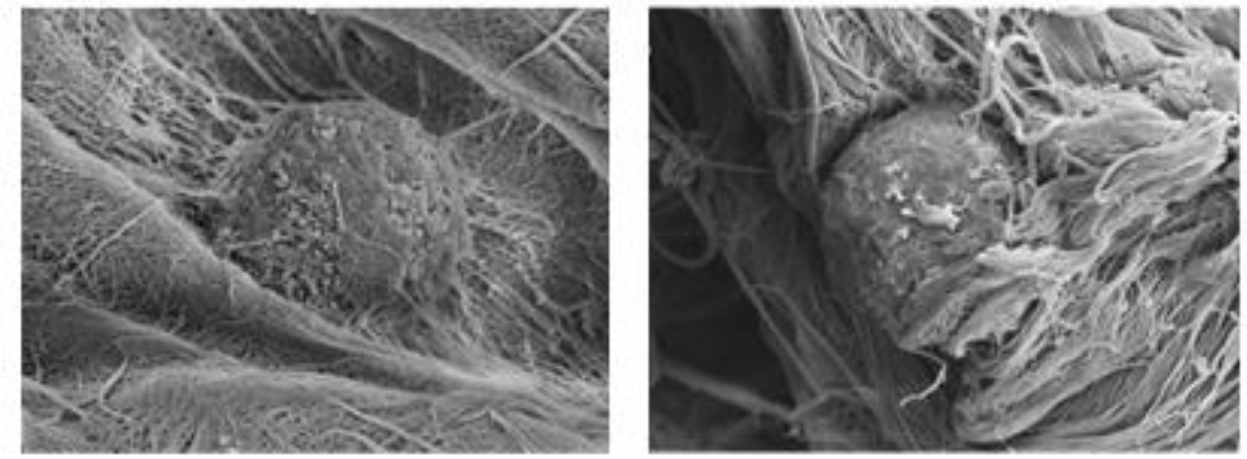


Figure 16: SEM images of HNP cells in crosslinked type II collagen-HA hydrogel (0.1 mM). Scale bar: 2 μm

4.1.8 Gene expression analysis by Real-time RT-PCR

The expression of type II collagen, type I collagen, Sox9, and aggrecan genes of HNP cells in the collagen-HA hydrogel were studied after they were cultured for 8 days or 30 days (Figure 17). The type II collagen gene expression of HNP cells cultured for 8 days in the gel crosslinked with 0.1 mM EDC (5.42 ± 1.28) was higher compared with that of cells cultured for 30 days (1.32 ± 0.59) (Figure 17A). The expression of type I collagen gene in HNP cells cultured

for 8 days in control gel (1.00 ± 0.11) or gel crosslinked with 0.1 mM EDC (0.22 ± 0.08) are significantly lower compared with that of HNP cells cultured for 30 days (8.95±2.75, control gel; 8.32 ± 2.38, crosslinked gel) (Figure 17B). The aggrecan gene expression of HNP cells cultured for 8 days (1.00 ± 0.12) in the control gel was significantly higher than that of HNP cells cultured for 30 days (0.36 ± 0.11) (Figure 17C). The Sox9 gene expression of HNP cells cultured for 8 days (1.00 ± 0.12) in the control gel was significantly higher than that of HNP cells cultured for 30 days (0.36 ± 0.11) (Figure 17D).

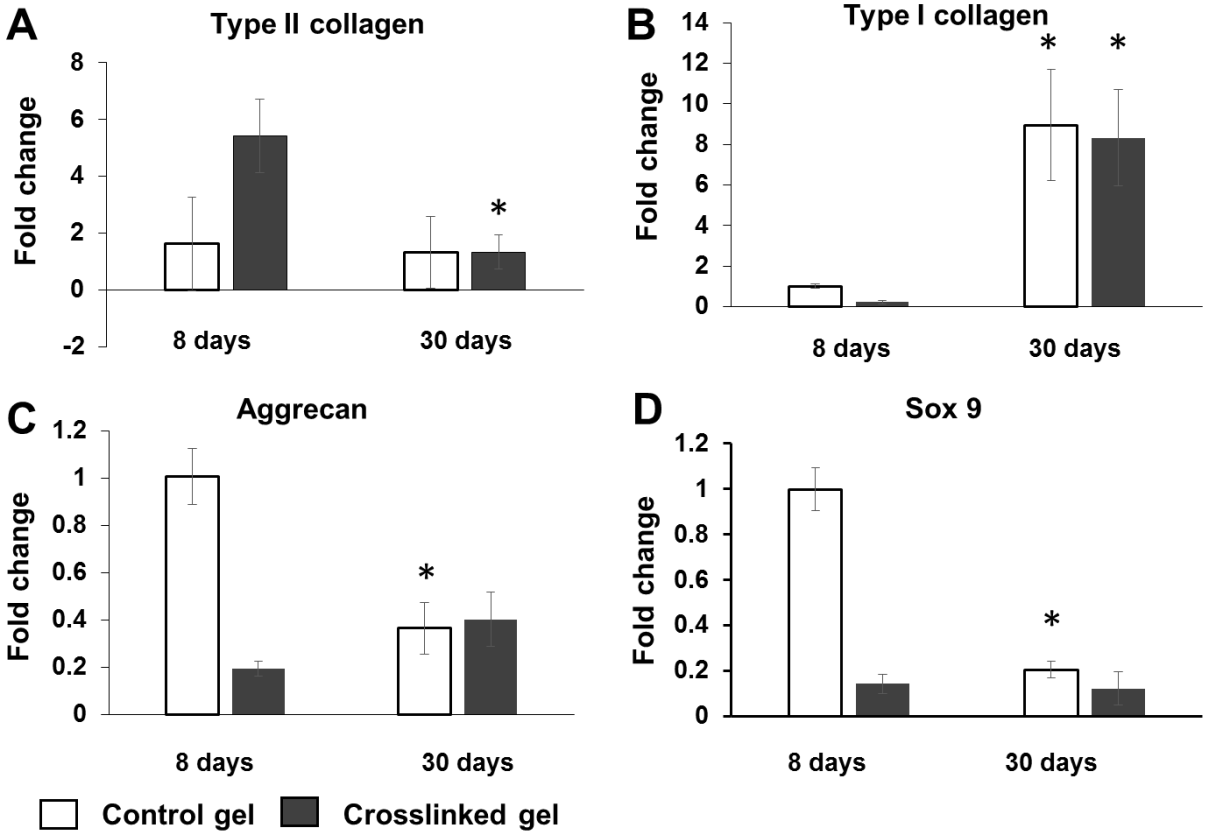


Figure 17: Results of qRT-PCR for chondrocytic cell marker genes Sox-9, type II collagen and aggrecan, and the fibroblastic marker gene type I collagen. Expressed as fold change compared with HNP cells in non-crosslinked type II collagen-HA hydrogel. Each target gene normalized to GAPDH (, $p < 0.05$, compared with corresponding result at 8 days).*

4.2 Discussion

In this study, we developed an injectable hydrogel composed of type II collagen and HA. We found that EDC-crosslinking of the hydrogel decreased the gel degradation rate caused by collagenase and reduced the uptake of PBS solution, compared with the non-crosslinked gel. We studied the degradation of the gel by collagenase at two time points. We showed that the crosslinked collagen-HA gel can be degraded by collagenase and increasing of the EDC amount reduced the gel degradation rate. Though the study cannot fully mimic in vivo process, it indicated that corsslinking enhanced the hydrogels and gels will be biodegradable in *in vivo* environment. The swelling study suggetsed that crosslinking can also stablilize the collagen-HA hydrogel and reduce water uptake after injection into the disc.

Natural biopolymers can mimic native ECM biochemistry and mechanical properties and therefore have been known to exhibit the ability to maintain cell viability. It was reported that alginate-based hydrogel is a suitable material for the proliferation of NP cells. After HNP cells were cultured for 21 days, the cell number was higher in the alginate hydrogel than in chitosan-gelatin scaffolds. In native NP tissue, since the ratio of type II collagen and HA is 9:1 (w/w), a hydrogel that mimics the native ECM constituents of NP tissue may produce an optimal environment for NP cell growth.[26] A previous study showed that porcine NP cells proliferated in the type II collagen-HA hydrogel.[11] However, the behavior of HNP cells in the type II collagen hydrogel has not been reported. In this study, we isolated NP cells from patients with degenerated IVDs and studied the cell viability in the type II collagen-HA hydrogel. While prior studies have shown the growth and biological behavior of cells in a biomaterial carrier, few studies have observed the cell retention and viability in the long-term culture of biomaterials. In this study, we found most HNP cells survived in the type II collagen-HA hydrogel and the hydrogel crosslinked with EDC (0.1 mM) after 2 months of cell culture. The NP cells showed an

elongated cell shape at the early stage of cell culture, and the cells developed multiple processes after culturing for 2 months in the hydrogel. The development of multiple processes suggested that the cells have become settled down and generated more contacts with ECM after long term cell culture. This observation suggests that the hydrogels provide a permissive environment for proliferation and maintenance of cell morphology of HNP cells.

Injectable materials that can generate minimal injury to animals have attracted considerable attention in IVD regeneration studies. Though natural materials allow the process of natural remodeling without the production of toxic byproducts, these biomaterials are not easily manipulated. Chemical crosslinking is a common way to enhance the physical and chemical properties of natural materials. EDC is a water-soluble zero-length crosslinker and has been shown to be less toxic than these bridge-linking crosslinkers. However, the toxicity of EDC is obvious at high level. Previous studies have shown that rat MSC can grow and proliferate in an EDC-crosslinked type II collagen-HA hydrogel.[12] The crosslinking of hydrogel with a high level of EDC (8 mM) significantly increased the stiffness of the gel. However, the hydrogel must be washed to remove EDC residues in the gel before the cells were seeded in the gel. In the procedure, it is difficult to evenly mix cells and the gel and repeated washing can also remove HA in the gel. In this study, we crosslinked the type II collagen-HA hydrogel with a low amount of EDC that can stabilize the gel and support human NP cell growth and proliferation. Because the toxicity of the gel is low, the mixture of the type II collagen-HA solution and cells can potentially be injected into the disc without a washing procedure. The hydrogel will form *in vivo* after injection and support NP cell growth.

The biological therapy approach attempts to regenerate degenerated tissue and accounts for a long-term cure by preventing or reversing the degeneration. The effective regeneration would rely on the remodeling of the ECM of NP tissue by the biological activity of

cells. A number of studies have been performed to investigate autologous NPC transplantation in animal models of degenerated tissue.[20,23,27] This approach has also been applied in clinical trials.[28] The application of biomaterial hydrogel that delivers therapeutic cells for the regeneration of NP provides the initial permissive environment for the cell to cope with the hostile environment. The laminin-111 functionalized poly(ethylene glycol) (PEG-LM111) hydrogel was developed as a biomaterial carrier for NP cell delivery to the IVD.[15] Poly(ethylene glycol) (PEG) hydrogels have been widely used in tissue engineering applications because of their non-toxic and hydrophilic properties.[29] The incorporation of biological signals such as a laminin peptide into the PEG significantly improved its biological function of serving as a carrier for NP cell delivery. Collagen type II is the ligand of cell membrane collagen receptors such as integrin β1. The interaction between receptors and the collagen type II mediates NP cell proliferation, migration, and adhesion. Additionally, the binding of HA with its cell membrane receptors may also mediate the cell phenotype response to the hydrogel.[30] A previous study showed that type II collagen-HA hydrogel crosslinked with 4-arm polyethylene glycol succinimidyl glutarate Mw10,000 (4S-StarPEG) were not toxic to bovine NP cells that grew in the gel.[11] However, the gene expression of major ECM components, such as type II collagen, type I collagen, and aggrecan, all decreased after culturing for 7 days, compared with cells at day 0. In this study, the gene expression of ECM for the cells in non-crosslinked gel and the gel crosslinked with low concentration of EDC (0.1mM) was studied, because the cells showed the better viability and morphology in these gels compared with the gels crosslinked with higher EDC (0.5mM and 1mM) in AlamarBlue assay and LIVE/DEAD cell viability assay.

In this study, we studied the cell viability and ECM gene expression at two different time points. The cells were cultured for a long time (2 months) to study the ability of the gels to support cell growth. Since the ECM gene expression of NP cells may change for a relatively

short time of culturing, it was measured after the cells were cultured for 1 month using quantitative reverse transcription polymerase chain reaction (qRT-PCR). We found though HNP cells proliferated in the type II collagen-HA hydrogel and the hydrogels maintained cell viability after culturing for 2 months, the qPCR assay showed the phenotypic drift of NP cells at earlier stage. The increased expression of the type I collagen gene was observed after the cells were cultured for 30 days compared with cells cultured for 8 days. We also observed a significant change of type II collagen gene expression of NP cells in the crosslinked gel (0.1mM EDC) and the aggrecan and Sox9 genes of NP cells in the non-crosslinked gel after a long-term cell culture. The results of gene expression studies may indicate the adaption of HNP cells to the 3D cell culture condition. The increased type I collagen expression may due to the cells from degenerated discs, which is a limitation for the study of NP cell function in the gel. Though the type II collagen-HA hydrogel provides a permissive condition for NP cell growth, mechanical stimulation may be necessary to maintain the cell phenotype and restore healthy NP tissue *in vivo*.

4.3 Conclusion

In this study, we fabricated an injectable type II collagen-HA hydrogel that is crosslinked with EDC, and studied the growth and gene expression of HNP cells in the hydrogel. We found that the optimal condition of the hydrogel for HNP cell growth is the non-crosslinked type II collagen-HA gel and the type II collagen-HA gel crosslinked with 0.1 mM EDC. Most cells in these hydrogels were survival cells and developed multiple processes after culturing for 2 months. A quantitative PCR study revealed the gene expression profile of the HNP cells in the hydrogels after culturing for 8 days and 30 days. The differential gene expression of the cells for short- or long-term culturing showed that the cells phenotype changed in the hydrogel. The change of gene expression of the cells may be the result of their adaption to the hydrogel at

different cell culture stages. Results of this study suggest that the type II collagen-HA hydrogel and the gel crosslinked with a low concentration of EDC are a suitable matrix for HNP cell growth and can serve as cell carriers for HNP cell transplantation into the IVD.

CHAPTER 5

CLINICAL RELEVANCE AND FUTURE DIRECTIONS

Injections of bioactive molecules and cells into the IVD are an effective technique to overcome the complications of disc degeneration. However, it is only effective in the early stages of disc degeneration. With pervasive cell damage and matrix loss, recovery from such extensive damages might not be possible through mere injections of biomaterials. Thus, in aberrant conditions of advanced stage degeneration, a total replacement of the disc tissues might be required. Under these circumstances, tissue engineering promises to be an excellent approach to grow the replacement disc tissue *in vitro* which can then be reimplanted into the patient's body *in vivo*. But more research and a better understanding is still required to develop a proper clinical practice and use these methods in the treatments of intervertebral disc degeneration.

More research groups are interested in the biology and application of tissue engineering towards solving the problems of disc degeneration. Research efforts mainly aim to develop effective biomaterials that are efficient and have no side effects and support the proper growth of replacement cells. But very few research initiatives have actually been successful and advanced to clinical trials. This low success rate can be attributed to the limited understanding of IVD cell phenotypes and their modulation during disc development, homeostasis, and disease.

A number of promising studies and investigations for the role of biomolecules and stem cells *in vitro* and *in vivo* using different animal models do exist. But there are still no successful reports where the biological conditions of human NP cells degeneration has are ideally mimicked. This may be due to differences in NP cell phenotype and load bearing capabilities among species. This is a major problem for the study of various possible methods that can be applied towards mitigating the effects of disc degeneration.

Our study provides a promising approach towards IDD therapy that can mimic the HNP. We generated an injectable hydrogel that can support human nucleus pulposus cell growth and proliferation and that can be used to further analyze the HNP phenotype and gene expression. Such analysis could include mechanical compression and shrinkage temperature so as to characterize and enhance the mechanical properties of the hydrogel.

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APPENDIX

APPENDIX

List of Abbreviations

bone morphogenetic proteins, BMPs; oeteogenic protein 1, OP1; tissue inhibitor of metalloproteinase TIMP-1; transforming growth factor, TGF; adeno-associated virus, AAV; human umbilical cord blood-derived mesenchymal stem cells, hUCB-MSCs; synovium-derived stem cells, SDSCs; adipose-derived stem cells, ADSCs; cartilage endplate-derived stem cells, CESCs; hyluronan-polyglycolic acid PGA; human-derived adipose tissue stromal cells, hADSCs; poly (L-lactic-co-glycolic acid), PLGA; chitosan–glycerophosphate, C/Gp; poly(ethylene glycol), PEG; intervertebral disc, IVD; annulus fibrosus, AF; nucleus pulposus, NP; extracellular matrix, ECM; hyaluronic acid, HA; 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide, EDC; N-hydroxy succinimide, NHS; mesenchymal stem cells, MSCs; link N nanofiber scaffold, LN-NS; chorioallantoic membrane, CAM; gellan gum, GG; ionic-crosslinked methacrylated GG, iGG-MA; magnetic resonance imaging, MRI; growth and differentiation factor 5, GDF_5; Fas-ligand (FasL; annulus fibrosus-derived stem cells, AFSCs; nucleus pulposus-derived stem cells, NPSCs; human telomerase reverse transcriptase, hTERT; keratin, type I cytoskeletal 19 (KRT19); adeno-associated virus serotype, AAV; adeno-associated virus serotype 2, AAV2; cartilage end plate, CEP; articular cartilage, AC; annulus fibrosis, AF; glycosaminoglycan side chains, GAGs; keratin sulphate, KS; and chondroitin sulphate, CS; Intervertebral disc degeneration, IDD; Self–assembling, SAP; extracellular matrix , ECM; Dulbecco's modified eagles medium, DMEM; fetal bovine serum, FBS; calcium chloride, $CaCl_2$; phosphate-buffered saline, PBS; ethidium homodimer-1, Ethd-1; scanning electron microscopy, SEM; quantitative reverse transcription polymerase chain reaction, qRT-PCR; PEG-LM111, laminin-111 functionalized poly(ethylene glycol, PEG; 4-arm polyethylene glycol succinimidyl glutarate Mw10,000, 4S-StarPEG;