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Phenotypic stability, matrix elaboration and functional maturation of nucleus pulposus cells encapsulated in photocrosslinkable hyaluronic acid hydrogels

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ABSTRACT

Degradation of the nucleus pulposus (NP) is an early hallmark of intervertebral disc degeneration. The capacity for endogenous regeneration in the NP is limited due to the low cellularity and poor nutrient and vascular supply. Towards restoring the NP, a number of biomaterials have been explored for cell delivery. These materials must support the NP cell phenotype while promoting the elaboration of an NP-like extracellular matrix in the shortest possible time. Our previous work with chondrocytes and mesenchymal stem cells demonstrated that hydrogels based on hyaluronic acid (HA) are effective at promoting matrix production and the development of functional material properties. However, this material has not been evaluated in the context of NP cells. Therefore, to test this material for NP regeneration, bovine NP cells were encapsulated in 1% w/vol HA hydrogels at either a low seeding density (20×10^6 cells ml^{-1}) or a high seeding density (60×10^6 cells ml^{-1}), and constructs were cultured over an 8 week period. These NP cell-laden HA hydrogels showed functional matrix accumulation, with increasing matrix content and mechanical properties with time in culture at both seeding densities. Furthermore, encapsulated cells showed NP-specific gene expression profiles that were significantly higher than expanded NP cells prior to encapsulation, suggesting a restoration of phenotype. Interestingly, these levels were higher at the lower seeding density compared to the higher seeding density. These findings support the use of HA-based hydrogels for NP tissue engineering and cellular therapies directed at restoration or replacement of the endogenous NP.

1. Introduction

Degeneration of lumbar intervertebral discs is strongly implicated as a cause of low back pain [1]. The central nucleus pulposus (NP) is critical for the mechanical function of the disc. The NP is a proteoglycan-rich gelatinous structure in the central region of the disc that is constrained circumferentially by the tough, fiber-reinforced annulus fibrosus (AF). When axial loads are applied to the spinal motion segment (bone–disc–bone unit), the NP is constrained from expanding by the AF, and so instead pressurizes, enabling even load transfer between adjacent vertebral bodies. Changes to the NP are an early hallmark of disc degeneration [2,3], and involve concurrent decreases in cell density, increases in inflammatory factors, fibrotic changes in tissue structure and an overall reduction in the synthesis of NP-specific extracellular matrix (ECM), especially proteoglycans [4]. During degeneration, this loss of NP-specific matrix content impairs NP mechanical function, decreasing swelling capacity and pressurization potential [5]. After the onset of degeneration, endogenous repair is limited due to the low cell density and poor nutrient supply of this avascular tissue [6].

Current treatments for disc degeneration, both conservative and surgical, have limited efficacy [7], and so there has been a strong focus on the development of new, biologic-based therapies that

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can restore and maintain native disc structure and mechanical function. To this end, tissue engineering efforts have sought to develop substitutes for the degenerated NP. Hydrogels in particular are among the most widely considered scaffold materials for NP tissue engineering as the native NP is a loosely crosslinked water swollen network (~90% water by weight), very similar in composition to many hydrogels used in biomedical applications [8]. Hydrogels for NP applications can either be used in an acellular form, including novel formulations that are "proteoglycan-rich" by design [9], or in combination with cells [8,10]. Importantly, hydrogel delivery can be performed minimally-invasively (i.e., percutaneously), and some formulations have been shown to restore aspects of native tissue composition and function in early translational studies [11]. A number of natural biopolymers have been considered for cell-based NP tissue engineering applications, including agarose [12,13], alginates that are both ionically and covalently crosslinked [10], carboxymethylcellulose [14], fibrin/fibrinogen [15], chitosan [16] and combinations thereof [17]. When encapsulated in these materials, NP and progenitor cells (i.e. mesenchymal stem cells) express NP-specific markers and accumulate NP-like ECM, including type II collagen and proteoglycans [13,18,19]. However, only a few studies have interrogated the mechanical properties of these constructs as a function of culture duration. For example, we recently showed that NP cells seeded in agarose can establish a functional NP-like material in vitro [13]. While promising, the translational potential and long term efficacy of agarose may be limited by its inability to degrade and be replaced by native tissue.

Over the last decade, we have explored the use of methacrylated hyaluronic acid (HA) as a cell carrier and hydrogel for the engineering of cartilaginous tissues using a variety of cell types, including chondrocytes and mesenchymal stem cells (MSCs) [20]. This material, when coupled with a photo-initiator, can form stable hydrogels upon UV exposure. Importantly, HA is used in many FDA-approved clinical procedures, and is biodegradable, increasing its translational potential [21,22]. Moreover, HA plays a critical role in the biology of the native NP matrix, where long chains of HA act as backbones for aggregating proteoglycans, contributing to the development of a functional ECM, while also evoking a positive biological effect on ECM production, cell migration and phenotypic maintenance of chondrocytes and NP cells [23]. When chondrocytes or MSCs are cultured in HA hydrogels under pro-chondrogenic conditions, these hydrogel constructs increase in mechanical functionality and ECM content with time [24]. More recently, we have shown that specific biologic interactions that cells have with HA via the CD44 receptor promote the early commitment of progenitor cells to the chondrogenic lineage, with associated positive effects for functional ECM deposition [25]. HA has also been used in combination with other materials to create composite hydrogels to take advantage of this biologic functionality. For example, Collin et al. developed an injectable type II collagen/HA hydrogel that promoted cell viability [26] while Park et al. fabricated fibrin/HA/silk composite gels that increased expression of type II collagen, sox9 and aggrecan, and maintained mechanical integrity in vitro [27]. Still more recently, Peroglio et al. developed a set of hyaluronan-based thermoreversible hydrogels (HA-pNIPAM) to serve as NP cell carriers, and cultured these cell-laden constructs for 1 week, with comparisons made to alginate gel beads. These HA-pNIPAM hydrogels maintained the NP cell phenotype and promoted ECM production [28].

In order to translate HA-based materials towards in vivo applications, it is important to consider the cell density and material attributes that will most effectively generate an NP-like tissue in the shortest possible time. One approach to improving the functional maturation of an NP cell-based engineered construct may be to increase the initial cell density within the construct. In the adult, the NP is matrix-rich and cell-poor, with a small number of endogenous cells (only ~6000 cells mm^{-3}, equating to ~6 million cells ml^{-1}). It is widely accepted that this low cell density is all that can be supported by the poor nutritional supply to the NP space [6]. However, during development, the NP begins essentially as a cell-rich, matrix-poor aggregation (i.e., its entirety is composed of cells prior to matrix deposition). A similar transition occurs in articular cartilage, where for example in fetal cartilage the cell density is as high as 100 million cells ml^{-1}, dropping to ~10 million cells ml^{-1} in the adult [29]. In both tissues, the high cellularity of the fetal state is thought to be required for the rapid accumulation and assembly of ECM, as well as its ability to repair itself. Indeed, this thinking informs many of our tissue engineering approaches, where for example increasing the seeding density of the engineered construct can improve growth trajectories [30]. Using the same seeding densities as were employed here (20 and 60 million cells ml^{-1}) with bovine mesenchymal stem cells in HA hydrogels (for cartilage tissue engineering purposes), higher cell densities resulted in improved functional outcomes with long term culture [24]. While these data point to a more rapid formation of an engineered construct for other cell types, this had not previously been assayed with NP cells in this HA system.

To that end, the objective of this study was to determine whether increasing the seeding density of NP cells in an HA hydrogel would enhance construct maturation. NP cells were seeded in 1% w/vol macromer density HA hydrogels at either 20×10^6 or 60×10^6 cells ml^{-1}, and cultured over an 8 week period in a chemically defined medium formulation. At multiple time points, construct maturation was evaluated by assessment of the biomechanical properties, expression of NP-specific genes, biochemical composition and ECM distribution to determine the appropriate NP seeding density for this HA-based NP tissue engineering system.

2. Materials and methods

2.1. Fabrication of hyaluronic acid (HA) hydrogels

Methacrylated HA (MeHA) was produced by reacting 65 kDa HA (Lifecore; Chaska, MN, USA) with methacrylic anhydride (Sigma Aldrich; St Louis, MO, USA) as previously described [21]. The degree of methacrylation was ~25%, as assessed by proton nuclear magnetic resonance as previously described [21]. Lyophilized MeHA was sterilized by exposure to a biocidal UV light for 15 min. Prior to cell encapsulation, the macromer was dissolved at 1% w/vol in sterile phosphate-buffered saline (PBS) with addition of 0.05% w/vol of the photoinitiator, Irgacure 2959 (2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone; Ciba-Geigy; Tarrytown, NY, USA).

2.2. Nucleus pulposus cell isolation, expansion, and three-dimensional (3-D) culture

NP tissue was isolated from four adult bovine caudal discs, purchased from a local slaughterhouse, according to institutional guidelines. NP cells (NPCs) were isolated from dissected tissue via digestion for 1 h in 2.5 mg ml^{-1} pronase, followed by 4 h in 0.5 mg ml^{-1} collagenase at 37 °C. After digestion, the cell suspension was filtered through a 70 μm strainer. Isolated NPCs were expanded in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin–fungizone (PSF). Passage 2 cells from all donor animals were combined and seeded into 1% w/vol MeHA solutions at a densities of either 20 million cells ml^{-1} (20 M) or 60 million cells ml^{-1} (60 M) as described previously [24].