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Collagen-low molecular weight hyaluronic acid semi-interpenetrating network loaded with gelatin microspheres for cell and growth factor delivery for nucleus pulposus regeneration

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ABSTRACT

Intervertebral disc (IVD) degeneration is one of the main causes of low back pain. Current surgical treatments are complex and generally do not fully restore spine mobility. Development of injectable extracellular matrix-based hydrogels offers an opportunity for minimally invasive treatment of IVD degeneration. Here we analyze a specific formulation of collagen-low molecular weight hyaluronic acid (LMW HA) semi-interpenetrating network (semi-IPN) loaded with gelatin microspheres as a potential material for tissue engineering of the inner part of the IVD, the nucleus pulposus (NP). The material displayed a gel-like behavior, it was easily injectable as demonstrated by suitable tests and did not induce cytotoxicity or inflammation. Importantly, it supported the growth and chondrogenic differentiation potential of mesenchymal stem cells (MSC) and nasal chondrocytes (NC) *in vitro* and *in vivo*. These properties of the hydrogel were successfully combined with TGF-β3 delivery by gelatin microspheres, which promoted the chondrogenic phenotype. Altogether, collagen-LMW HA loaded with gelatin microspheres represents a good candidate material for NP tissue engineering as it combines important rheological, functional and biological features.

1. Introduction

Low back pain is increasingly burdening the health care system and economy in industrialized countries. Between 50% and 80% of the population experience symptoms of low back pain [1]. The reasons for the pain syndrome are multiple, with intervertebral disc (IVD) degeneration being one of the main causes [2]. IVD is a cartilaginous tissue located between neighboring vertebras of the spine. Its key functions include stress absorption during movement and providing the spine with a certain degree of motility. IVD consists of an outer part – the annulus fibrosus (AF) and an inner part – the nucleus pulposus (NP). AF is an acellular fibrous tissue with a high content of type I collagen. NP, in contrast, is a gel-like tissue resembling hyaline cartilage containing mainly type II collagen and sulfated glycosaminoglycans (GAG) that form the extracellular matrix (ECM) for the so-called NP cells [3]. Both AF and NP are mostly avascular with the supply of oxygen and nutrients occurring via diffusion from blood capillaries in the outer AF and, most importantly, the cartilaginous endplate (EP), which connects IVD to bone tissue [4]. Age-related calcification of EP in addition to physical wearing of IVD, trauma, environmental, genetic and other factors can lead to IVD degeneration [5]. This pathology is often associated with IVD thinning, bulging and herniation, all of which can cause pain [3].

Current therapy of low back pain includes analgesics, anti-inflammatory drugs and physiotherapy. More complex cases of IVD degeneration demand surgical treatment, such as discectomy, spinal fusion and total disc replacement [6]. In these surgical approaches a part of or an entire IVD is removed to relieve the pain, the remaining cavity being filled with bone grafting material to promote the fusion of adjacent vertebrae or, alternatively, with a total disc implant. However, such treatments often do not restore full mobility of the spine and can even lead to the degeneration of the neighboring IVD [7]. As a potential alternative approach,

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tissue engineering of IVD aims at rebuilding native IVD structure by combining appropriate biomaterials with cellular regenerative potential. IVD is an anatomically complex structure and different tissue engineering approaches have been proposed for the regeneration of different IVD compartments [3].

High water content and the gel-like structure of NP encourage the use of hydrogels in NP tissue engineering. Such hydrogels could be injected into the NP, completely fill the defect in a liquid state and after polymerization provide adequate mechanical strength to the IVD. In fact, several hydrogels have been developed for this purpose [8,9]. Injectable hydrogels offer a possibility for minimally invasive treatment of IVD degeneration. Hydrogels made of the natural ECM components present several advantages, such as good tissue compatibility, viscoelastic properties and structures similar to the native ECM. Although such materials alone can restore the mechanical properties of NP, a combination of hydrogels with cells and/or growth factors could promote IVD regeneration and in some cases it has been shown to be more effective than hydrogels alone [10]. The rationale behind this approach is the production of the ECM molecules by implanted or native cells, which in the course of time will replace the injected hydrogel, while growth factors are expected to boost ECM deposition. Several variations of this strategy, such as the use of cells or growth factors alone, have been tested [11,12].

NP cells could be envisaged as the cell source for NP regeneration. However, their availability and *in vitro* expansion capacity is limited. NP cells from degenerated IVD usually available for isolation might have an altered phenotype [13]. On the other hand, NP biopsy from a healthy IVD might cause irreparable damage to the AF and induce IVD degeneration. Therefore, other cell sources with regenerative potential would be highly useful for NP tissue engineering. Mesenchymal stem/stromal cells (MSC) are of interest due to their multilineage differentiation potential [11]. MSC can be easily isolated from e.g. bone marrow or adipose tissue and directed to differentiate along the chondrogenic lineage [14]. Moreover, differentiated chondrocytes could be used for NP regeneration, although their potential has yet to be demonstrated *in vivo*. In this respect, nasal chondrocytes (NC) represent a good cell type for tissue engineering due to easy accessibility of nasal cartilage tissue [15]. Both differentiated MSC and chondrocytes produce ECM, similar to NP components, such as GAG and type II collagen. Differentiation and ECM deposition can be stimulated *in vitro* with several growth factors, such as TGF-β3 [14]. Alternatively, growth factors could be injected into the NP, where they would induce a chondrogenic phenotype in co-injected cells or in native NP cells [12]. In yet another approach, the cells used for NP regeneration could be genetically modified to produce high levels of chondrogenic growth factors [16]. However, this strategy is associated with a number or regulatory hurdles.

Several acellular and cell-loaded hydrogels for NP tissue engineering have been developed [8,17,18]. However, the search for the most optimal cell/growth factor-loaded material continues, and patients have yet to experience the benefit from extensive research in IVD tissue engineering. In this study we analyzed a specific formulation of collagen-low molecular weight hyaluronic acid (LMW HA) semi-interpenetrating polymer network (semi-IPN) loaded with gelatin microspheres as a promising candidate for NP substitute material. It is hypothesized that this material is able to mimic the gel-like rheological behavior of the natural NP and at the same time support the growth and chondrogenic potential of relevant cell types, such as MSC and NC, while gelatin microspheres could be used for growth factor delivery. Viscoelastic properties, flow behavior, functional injectability of the proposed composite hydrogel-based system, as well as its biologic performance *in vitro* and *in vivo*, were assessed in this study.

2. Materials and methods

2.1. Collagen-LMW HA semi-IPN loaded with gelatin microspheres

Collagen-LMW HA semi-IPN were obtained by promoting collagen fibrillogenesis in the presence of LMW HA. Sterile LMW HA powder (M_w = 150–300 kDa, ANIKA THERAPEUTICS, Abano Terme, Italy) dissolved in DMEM was suitably added to sterile collagen solution (Vitrogen) to provide a final LMW HA concentration of 2.5 mg/mL and collagen concentration of 1.2 mg/mL. Hydrated and autoclaved Cultispher-G microspheres (Percell) were added to the mixture to the final concentration of 0.25 mg/mL. Furthermore, all steps were performed under sterile conditions. The solution was incubated at 37 °C for 1 h to promote collagen fibrillogenesis. After the incubation the hydrogel-based composite system was obtained.

2.2. Rheological analyses and injectability tests

The viscoelastic properties of collagen-LMW HA semi-IPN were evaluated at 37 °C using a rheometer (Gemini, Bohlin Instruments) with parallel-plate geometry. Specifically, serrated parallel plates (15 mm in diameter) with a gap of 1 mm were used to avoid slippage. Strain sweep tests were carried out to determine the linear viscoelastic region, then small amplitude oscillatory shear tests were performed. The frequency was varied from 0.01 to 2 Hz. The storage modulus or elastic modulus (G') and loss modulus or viscous modulus (G'') were evaluated in the frequency range investigated as follows:

$$G' = \frac{\tau_0}{\gamma_0}\cos\delta$$

$$G'' = \frac{\tau_0}{\gamma_0}\sin\delta$$

where δ is the phase shift between the input and the output signals, while τ_0 and γ_0 represent stress and strain amplitudes. Specifically, small amplitude oscillatory shear tests were performed on collagen-LMW HA semi-IPN before and after injection through a 16 G needle.

The viscosity as a function of the shear rate was evaluated through steady shear measurements carried out at a temperature of 37 °C over a wide range of shear rates (0.01–50 s^{-1}).

The injectability was also analyzed at 37 °C using a syringe with a 4.5 mm inner diameter and a 16 G needle. The syringe was loaded with the material and suitably mounted on an INSTRON 5566 testing machine. The piston was driven at a speed of 40 mm/min and the applied load for injecting the materials into and through the 16 G needle was measured. An empty syringe was also tested to provide information on the friction between the syringe walls and the piston.

2.3. MSC isolation

MSC were isolated from bone marrow following the protocol adopted from Horn et al. [19]. The tissue was collected according to the guidelines of the Ethics Committee of the State of the Rheinland-Palatinate. Bone marrow was pre-diluted with PBS, mixed with Red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2–7.4) in a ratio 1:9 and incubated at room temperature for 3 min. After centrifugation for 3 min at 1500 rpm and subsequent washing in PBS, MSC were seeded on cell culture plastic in DMEM-F12 (Gibco) supplemented with 10% FCS (Sigma) and 1% Penicillin/Streptomycin solution (Gibco). The cells were cultivated at humidified atmosphere with 5% CO_2 at 37 °C and used in passage 2–6.