2017

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Wachs, Rebecca A.; Hoogenboezem, Ella N.; Huda, Hammad I.; Xin, Shangjing; Porvasnik, Stacy L.; and Schmidt, Christine E., "Creation of an injectable in situ gelling native extracellular matrix for nucleus pulposus tissue engineering" (2017). *Biological Systems Engineering: Papers and Publications*. 505.
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FDA device/drug status: Not applicable. **Author disclosures:** **RAW:** Support for Travel to Meetings for the Study or Other Purposes: BMES Career Development Award (A), pertaining to submitted manuscript. **ENH:** Nothing to disclose. **HIH:** Nothing to disclose. **SX:** Nothing to disclose. **SLP:** Nothing to disclose. **CES:** Grants: NSF (Hydrogels for peripheral nerve regeneration) (F, Paid to the institution), DARPA (Peripheral nerve electronic interfaces) (E, Paid to the institution), outside the submitted work. The disclosure key can be found at www.TheSpineJournalOnline.com.

Abstract

Background Context: Disc degeneration is the leading cause of low back pain and is often characterized by a loss of disc height, resulting from cleavage of chondroitin sulfate proteoglycans (CSPGs) present in the nucleus pulposus. Intact CSPGs are critical to water retention and maintenance of the nucleus osmotic pressure. Decellularization of healthy nucleus pulposus tissue has the potential to serve as an ideal matrix for tissue engineering of the disc because of the presence of native disc proteins and CSPGs. Injectable in situ gelling matrices are the most viable therapeutic option to prevent damage to the annulus fibrosus and future disc degeneration.

Purpose: The purpose of this research was to create a gentle decellularization method for use on healthy nucleus pulposus tissue explants and to develop an injectable formulation of this matrix to enable therapeutic use without substantial tissue disruption.

Study Design: Porcine nuclei pulposi were isolated, decellularized, and solubilized. Samples were assessed to determine the degree of cell removal, matrix maintenance, gelation ability, cytotoxic residuals, and native cell viability.

Methods: Nuclei pulposi were decellularized using serial detergent, buffer, and enzyme treatments. Decellularized nuclei pulposi were solubilized, neutralized, and buffered. The efficacy of decellularization was assessed by quantifying DNA removal and matrix preservation. An elution study was performed to confirm removal of cytotoxic residuals. Gelation kinetics and injectability were quantified. Long-term in vitro experiments were performed with nucleus pulposus cells to ensure cell viability and native matrix production within the injectable decellularized nucleus pulposus matrices.

Results: This work resulted in the creation of a robust acellular matrix (>96% DNA removal) with highly preserved sulfated glycosaminoglycans (>47%), and collagen content and microstructure similar to native nucleus pulposus, indicating preservation of disc components. Furthermore, it was possible to create an injectable formulation that gelled in situ within 45 minutes and formed fibrillar collagen with similar diameters to native nucleus pulposus. The processing did not result in any remaining cytotoxic residuals. Solubilized decellularized nucleus pulposus samples seeded with nucleus pulposus cells maintained robust viability (>89%) up to 21 days of culture in vitro, with morphology similar to native nucleus pulposus cells, and exhibited significantly enhanced sulfated glycosaminoglycans production over 21 days.

Conclusions: A gentle decellularization of porcine nucleus pulposus followed by solubilization enabled the creation of an injectable tissue-specific matrix that is well tolerated in vitro by nucleus pulposus cells. These matrices have the potential to be used as a minimally invasive nucleus pulposus therapeutic to restore disc height.

Keywords: Decellularized matrix, Disc degeneration, Injectable scaffold, Intervertebral disc, Nucleus pulposus, Tissue engineering

Introduction

Low back pain will affect 70% to 80% of the population at some point in their lifetime, and disc degeneration is the leading cause of low back pain [1]. Disc degeneration is characterized by a loss of intervertebral disc (IVD) height, reduced water content, end plate thickening, and annular fissures [2–7]. Breakdown of the nucleus pulposus, a gelatinous matrix rich in collagen type II and chondroitin sulfate proteoglycans (CSPGs) [8,9], has been implicated in disc degeneration [10]. The CSPGs have sulfated glycosaminoglycan (sGAG) side chains that attract water, enabling the nucleus to withstand substantial compressive loads and prevent nerve ingrowth [8,9,11]. Cleavage of the CSPGs and consequent loss of sGAG side chains have been implicated in disease progression by resulting in the loss of the ability of the nucleus to hold water and therefore bear compressive loads [10]. The loss of CSPGs may additionally enable innervation of the disc and subsequent pain [11,12]. Discogenic pain is one source of low back pain that is thought to arise from increased nociceptive fiber innervation in the IVD [13,14].

A variety of approaches are currently being investigated to repair the nucleus pulposus and prevent low back pain from occurring. Tissue engineering approaches often combine growth factors and cells to attempt to repair the nucleus pulposus; however, in the harsh catabolic environment of the degenerating disc, growth factors and cells cannot survive [15–20]. The addition of a biomaterial carrier has the potential to increase cell viability and enable long-term growth factor delivery. Various synthetic and natural materials are currently being investigated for nucleus pulposus tissue engineering [21–26]. However, synthetic materials, such as chemically modified hyaluronan, can have toxic byproducts or result in a foreign body response [27–29].

An ideal solution for tissue engineering of the nucleus pulposus would (1) mimic the native disc composition and consist of a proteoglycan rich matrix to restore the hydrostatic compressive resistance of the disc, (2) serve as a cell carrier, (3) prevent innervation into the nucleus, and (4) be deliverable via injection and in situ gelation. Decellularized nucleus pulposus tissue has the potential to be this ideal scaffold because it is composed of native disc proteins and proteoglycans with similar structure to native disc. In addition, a decellularized scaffold has the potential to be developed into an injectable formulation that gels in situ.

Tissue-derived biomaterials can offer unique advantages over synthetic materials such as limited foreign body response, native cell response, and integration into

surrounding tissue using endogenous enzymes [30–33]. However, to minimize immune rejection of the tissue and to prevent the need for systemic immunosuppression, it is essential to remove cells and antigens. Decellularization of tissue is a practice that has been used for decades to create naturally derived scaffolds. Removal of cells and antigens is essential to preventing an immune response; however, decellularization must also maintain the composition and structure of the native tissue [33–36]. Decellularization techniques have been optimized for the heart [37], lung [38], nerve [39], skin [40], brain [41], and spinal cord [42], among other tissues, and can involve physical, chemical, and biological methods to lyse cells, and subsequently remove cellular debris. Our laboratory has previously developed gentle decellularization techniques for nerve tissue to preserve essential proteins, proteoglycans, and microstructure [39,43], which would be ideal for the delicate nucleus pulposus. To date, some research has been performed to create decellularization protocols for the nucleus pulposus [44–47], annulus fibrosus [48,49], and the intact IVD [50]. These matrices have shown promise as viable scaffolds for IVD cells as well as drivers of stem cell fate [44–50], although they are not injectable or in situ gelling.

After a tissue has been decellularized, it is possible to turn the tissue into an injectable in situ gelling biomaterial [37,51], which would enable an easier therapeutic delivery with minimally invasive surgery. For IVD, an injectable in situ gelling material would support delivery through a fine gauge needle with no disruption of the annulus fibrosus, while also enabling formation of a gel capable of supplementing load bearing of the nucleus pulposus. Works by Freytes et al. and Singelyn et al. document the early processes to solubilize decellularized urinary bladder matrix and heart tissue, enabling in situ gelation by using pepsin in hydrochloric acid [37,51]. Kwon et al. used pepsin in acetic acid to solubilize cartilage [52]. Others have used modifications of these protocols to solubilize a variety of tissues, including brain [41], liver [53], and tendon [54]. However, these protocols have not yet been adapted to create injectable in situ gelling hydrogels from decellularized nucleus pulposus or IVD tissue.

The purpose of this research was to create a gentle nerve decellularization method for use on the nucleus pulposus tissue of the IVD, thereby creating a tissue-specific acellular matrix with increased maintenance of proteoglycans and sGAGs with the potential to prevent nerve ingrowth and drive stem cell fate. In addition, we also wanted to create an injectable formulation of this matrix to enable therapeutic use without substantial disruption of the annulus fibrosus.

Fig. 1. Macroscopic image of lumbar spine segment after cleaning (Left) and dissection (Right).

Materials and methods

Nucleus pulposus harvest and processing

Whole spines were dissected *en bloc* from Yorkshire-Landrace porcine weighing 40 to 50 kg (Fig. 1). Excess tissue was removed and spines were disarticulated into cervical, thoracic, and lumbar segments. Spine segments were stored at -80°C until use. Spine segments were then thawed for 24 hours, and the nuclei pulposi were isolated from the cervical, thoracic, and lumbar sections of the spine using careful dissection, and stored for up to 1 hour in sterile $1\times$ phosphate buffered saline (PBS) until use (Fig. 2A, B, and C).

The nuclei pulposi were decellularized using a gentle technique adapted from Hudson et al. previously developed in our laboratory. All samples were processed in 15 mL tubes in an orbital shaker at 15 rpm. The samples were immersed in water for 7 hours, followed by 100 mM sodium and 50 mM phosphate buffer for 10 hours, SB-10 detergent for 4 hours, 100 mM sodium and 50 mM phosphate buffer for 15 minutes, Triton X-200/SB-16 for 3 hours, 100 mM sodium and 50 mM phosphate buffer

for 3×15 minutes, SB-10 detergent for 1.75 hours, 100 mM sodium and 50 mM phosphate buffer for 15 minutes, Triton X-200/SB-16 for 3 hours, 100 mM sodium and 50 mM phosphate buffer for 3×15 minutes, and followed by DNase/RNase solution (75 U/mL/100 μg /mL) for 34 hours. The decellularized nuclei pulposi (dNPs) were then washed in 50 mM sodium and 10 mM phosphate buffer for 3×90 min. Macroscopic images of nuclei pulposi before and after decellularization are illustrated in Fig. 2.

DNA removal and sGAG maintenance

DNA removal and sGAG maintenance were determined using quantitative assays. Fresh nucleus pulposus (NP) control and dNP samples were fixed in 4% paraformaldehyde (PFA) for 30 minutes and washed 3×15 minutes in $1\times$ PBS. Of each sample, 50 mg wet weight was cut and digested in papain (4762, Sigma, St Louis, MO, USA) at 65°C for 18 hours. After digestion, DNA was analyzed using a Quant-IT PicoGreen assay (P7589, Thermo Fisher, Waltham, MA, USA). The sGAG content was quantified using a Blyscan assay (Biocolor, Carrickfergus, Ireland). A total of six dNPs were quantified from the cervical,

Fig. 2. Macroscopic images of nuclei pulposi from the cervical, thoracic, and lumbar spine segments, respectively, before (A, B, C) and after (D, E, F) decellularization.

thoracic, and lumbar segments of three distinct spines and compared with matched fresh NP controls.

Collagen content

The collagen content of fresh NP control and dNP samples was assessed using both soluble and insoluble collagen assays. Fifty milligrams of fresh NPs and dNPs were digested in 1 mg/mL pepsin (P7012, Sigma) in 0.5M acetic acid at 4°C for a minimum of 18 hours on a shaker plate. After digestion, the soluble collagen assay was executed followed by the insoluble assay using standard assay kits (CLRS1000, CLRS2000, Biocolor). The collagen content was normalized to wet weight. A total of six dNPs were quantified from three distinct spines and compared with six fresh NP controls.

Cell removal and maintenance of CSPGs

Cell removal and maintenance of CSPGs after decellularization were qualitatively assessed using fluorescence staining and confocal imaging. Decellularized and fresh NP samples were fixed in 4% PFA for 30 minutes, washed 3 × 15 minutes in 1× PBS, and permeabilized with 4% goat serum and 0.5% Triton X-100 in 1× PBS. Following permeabilization, dNP samples were stained overnight with a primary antibody to CSPGs (C8035, 1:200, Sigma) in blocking buffer containing 0.3% Triton X-100 (93443, Sigma) and 3% goat serum (G9023, Sigma) in 1× PBS. An Alexa Fluor 568 secondary antibody (A11031, 1:500, Thermo Fisher) was used to fluorescently label the CSPG primary. All samples were then counterstained with DAPI (D1306, Thermo Fisher) for 5 minutes and washed 3 × 5 minutes in 1× PBS. A Zeiss LSM 710 confocal microscope (Zeiss Microimaging Inc., Jena, Germany) was used to take z-stacks through each NP sample. Maximum intensity projections were created through z-thickness matched samples to enable semi-quantitative analysis.

Assessment of cytotoxic residuals

Decellularized NP samples were eluted for 72 hours at 37°C in 10 mL of medium containing 89% DMEM:F12 (D8437, Sigma), 10% fetal bovine serum (16000044, Life Technologies, Carlsbad, CA, USA), and 1% penicillin-streptomycin (51606, EMD Millipore, Billerica, MA, USA). A total of 16 dNPs were eluted from four distinct spines (n=3–6 per spine). After 72 hours, eluted medium from each sample was removed and 250 μ L was added to a 48-well plate containing ~7,500 human neonatal dermal fibroblasts (PCS-201-010, ATCC, Manassas, VA, USA) in triplicate per sample. Control wells containing fresh medium were also cultured (n=3). After 48 hours, a metabolic assay (DAL1100, Thermo Fisher) was performed to assess the metabolic activity of eluted dNP samples medium versus control medium and determine if cytotoxic residuals were present.

Creation of an injectable dNP matrix

In preparation for solubilization, dNPs were washed three times in ddH₂O and then lyophilized for 72 hours. After lyophilization, dNPs were cut in small pieces and pooled for digestion (Fig. 5B). Chopped dNPs were suspended at 5 mg to 20 mg/mL in 1 mg/mL pepsin in either 3% acetic acid or 0.01 M hydrochloric acid with or without homogenization for 3 × 30 seconds. dNPs were then placed on a stir plate at room temperature for 32 to 64 hours to facilitate digestion. After 32 to 64 hours, each sample was neutralized using either 0.1M or 1M sodium hydroxide and buffered with 10% of 10× sterile PBS. The ability to thermally gel was tested by pipetting 10 to 30 μ L of solubilized dNPs onto paraffin and incubating for 45 minutes at 37°C. Solubilized dNPs were stored at 4°C for up to 7 days until use.

Gelation kinetics

Thermal gelation of collagen occurs through fibrillar formation and crosslinking. As this process occurs, proteins aggregate and form cross-links and the absorbance of 405 nm light increases. This phenomenon can be used to determine gelation kinetics by examining the temporal increase and plateau in absorbance as gelation progresses to completion. To assess gelation kinetics, 50 μ L of chilled pre-gel solubilized dNP was placed into a 96-well plate and absorbance was measured at 405 nm 37°C using the Synergy HT Plate Reader (BioTek, Winooski, VT, USA). Four batches of injectable dNPs were tested in duplicate. Absorbance readings were recorded every 2 minutes over a total of 60 minutes. Type I collagen gels were used as a positive control, and a solution of 3% acetic acid neutralized with 5 M sodium hydroxide and buffered with 10% 10× PBS was used as a negative control. Data were normalized to the initial readings using Equation (1), where A is the absorbance, A_0 describes the initial absorbance, and A_{max} is the maximum absorbance.

$$\text{Normalized absorbance} = \frac{A - A_0}{A_{max} - A_0} \quad (1)$$

Injectability of solubilized dNP matrix

To test the ability of the solubilized dNPs to be injected and still form a gel, the samples were ejected through a 30 gauge needle using a maximum force of <200 N, which is the maximum applied force for injection by hand [55]. After injection, the ability to thermally gel was tested by incubating for 45 minutes at 37°C.

Microstructure

Decellularized, solubilized and re-gelled, solubilized injected and re-gelled, and fresh NP samples were each prepared for scanning electron microscopy. The samples were fixed in 4% PFA for 30 minutes, washed 3 × 15 minutes in 1× PBS, and then subjected to serial ethanol

dehydration (30%, 50%, 70%, 85%, 90%, 95%, and 100%) over the course of 2 hours. After dehydration, samples were dried using serial solutions of hexamethyldisilazane (25%, 50%, 75%, and 100%) over the course of 2 hours. The samples were then left to dry overnight in a fume hood in hexamethyldisilazane. High-resolution scanning electron microscopy (SEM) was performed using a Nova NanoSEM (FEI, Hillsboro, OR, USA). Images were acquired at magnifications ranging from 5,000 $\times$ to 80,000 $\times$. ImageJ [56,57] was used to quantify fiber diameter at 20,000 $\times$ and assess maintenance of structure in decellularized, solubilized and re-gelled, solubilized injected and re-gelled, and fresh NP samples. A minimum of five images per group were quantified, and a minimum of 175 collagen fibers per image were measured.

NP cell-seeded constructs

Human nucleus pulposus cells (#4800, ScienCell Research Laboratories, Carlsbad, CA, USA) were cultured and passaged in complete medium containing 1% penicillin-streptomycin, 10% fetal bovine serum, and 1% nucleus pulposus cell growth supplement (#4801, ScienCell Research Laboratories) at 37°C and in normoxic conditions. Nucleus pulposus cells from passage 1 to 3 were gently lifted using trypsin-EDTA (25200056, Thermo Fisher) and mixed with solubilized dNP samples. Solubilized dNPs and cell mixtures were pipetted onto parafilm in 30 μ L droplets (40,000 cells per gel) and incubated for 45 minutes at 37°C to allow for thermal gelation. After gelation, samples were placed in a 48-well plate and immersed in 200 μ L of complete medium. Two batches of dNPs were cultured for 1, 7, or 21 days under hypoxic conditions of 3.5% oxygen and 5% carbon dioxide to mimic conditions present in *in vivo* nucleus pulposus (n=6 per time point per batch).

Viability, sGAG content, and collagen content were determined at each time point. A standard live-dead assay using ethidium homodimer and calcein AM (L3224, Thermo Fisher) was performed at days 1, 7, and 21 to determine cell viability in gels. After staining, confocal imaging was used to image intact gels (LSM 710, Zeiss Microimaging Inc.). A minimum of three 10 $\times$ images were

taken for each gel and quantified using Zeiss ZEN ImageAnalysis software (Zeiss Microimaging Inc.). Z-stack images were also acquired to assess cell morphology in gels. Sulfated glycosaminoglycan and collagen content were analyzed using the methods listed in the *DNA Removal and sGAG Maintenance* section.

Statistics

Paired students *t* tests were performed to assess differences between dNPs and fresh NP controls ($p < .05$) for the sGAG quantification, DNA content, collagen content, metabolic activity assessment of cytotoxic residuals, and SEM fiber diameters. In addition, an analysis of variance with Tukey pairwise comparison was used to determine differences in viability, sGAG content, and DNA content between time points and batches in the nucleus pulposus cell-seeded construct experiment.

Results

DNA, sGAG, and collagen content

The results indicate that it was possible to create a gentle decellularization procedure for porcine nucleus pulposus. Over 96% of DNA content was removed in dNPs from cervical, thoracic, and lumbar segments (Fig. 3, Left). In addition, sGAG preservation was 44.5%, 21.9%, and 50.8% for dNPs from cervical, thoracic, and lumbar segments, respectively, as shown in Fig. 3, Middle. Because of the low sGAG preservation in the thoracic discs, all following studies exclusively used cervical and lumbar discs for processing and testing. The total collagen content was maintained after decellularization, with dNP samples exhibiting a total collagen content of 18.6 μ g of collagen per milligram wet weight and control NP samples exhibiting 16.12 μ g/mg (Fig. 3, Right). There was no significant difference between the two groups.

Cell removal and maintenance of CSPGs

Confocal imaging revealed faint DAPI staining in the majority of dNPs, with some containing no areas with any DAPI staining. After decellularization, CSPGs were well

Fig. 3. DNA content was significantly removed in decellularized nuclei pulposi (dNPs) versus fresh nucleus pulposus (NP) controls, with greater than 96% DNA being removed in all samples (Left) ($p < .05$). Sulfated glycosaminoglycans were well maintained (>45%) in the cervical and lumbar nuclei pulposi (Middle) ($p < .05$). Concentrations of soluble, insoluble, and total collagen were not significantly different between the control NP and dNP samples (Right). Data (n=6) represent the mean $\pm$ standard error.

Fig. 4. Cell nuclei were removed (DAPI, left panels, blue) and chondroitin sulfate proteoglycans (CSPGs, right panels, red) were maintained after decellularization of nuclei pulposi (Bottom) compared with fresh nuclei pulposi controls (Top).

preserved throughout the matrix. Fig. 4 is a representative image of a control nucleus pulposus (Top) and a dNP (Bottom). No differences were observed between the spine segments (cervical and lumbar); however, it was not possible to quantify staining because of high variability in cell density in different areas of the nucleus pulposus.

Cytotoxicity

The metabolic activity of human neonatal dermal fibroblasts with eluted dNP sample medium was not significantly lower than the control medium for any of the samples tested. Across all samples tested, the average difference in metabolic activity compared with the control medium was $1.11\% \pm 2.1$ standard error. This indicates that there are no cytotoxic residuals remaining from the decellularization process that impact cell function.

Creation of an injectable dNP matrix

The optimal parameters for the creation of a robust injectable dNP matrix were achieved by solubilizing minced dNPs at 20 mg/mL in 1 mg/mL pepsin in 3% acetic acid, homogenizing for 3×30 seconds, and stirring at room temperature for 32 hours before neutralization and

buffering (Fig. 5). These gels could be manually manipulated and did not break apart when submerged in $1\times$ PBS for up to 7 days.

Gelation kinetics

Stabilization of absorbance of all four batches of dNP samples occurred by ~ 40 minutes, indicating near complete gelation. A slope of 0 was observed in all samples by 60 minutes, indicating complete gelation. Type I collagen controls (354236, Corning, Corning, NY, USA) exhibited complete gelation in 20 minutes.

Injectability of solubilized dNP matrix

The solubilized dNP samples were easily injected through a 30 gauge syringe with minimal manual pressure. No macroscopic disruption of the matrix was observed, and they were able to form a smooth gel surface. Samples gelled in ~ 40 minutes, similar to non-injected solubilized dNP controls. SEM was used to assess any changes in microstructure.

Microstructure preservation

SEM revealed excellent preservation of microstructure after decellularization, and solubilization and re-gelation

Fig. 5. Pictorial illustration of the digest and re-gelation process of the decellularized nuclei pulposi, from post-lyophilization (A), to fine chop (B), to homogenization (C), and to post-gelation (D).

of NP samples (Fig. 6A, B, and C). The collagen fibrils were intact and not substantially disrupted. Furthermore, no cellular debris was apparent in dNPs. Although fiber diameter was significantly increased after decellularization compared with controls, and significantly decreased after solubilization compared with controls, both diameters were within 10% of the control NP fiber diameter (Fig. 6D). The microstructure was also assessed after injection through a fine gauge needle and regelation of the dNPs. There was no significant difference in fiber diameter of solubilized dNPs that were gelled without injection compared with those that were solubilized, injected, and then gelled.

NP cell-seeded constructs

Nucleus pulposus cells maintained excellent viability (>89%) over the entire 21-day culture period, as illustrated in Fig. 7, Left, with a significant increase observed between day 1 and day 7, and no significant

differences observed between day 1 or day 7 and day 21. In addition, by day 21, NP cells exhibited an elongated morphology similar to that observed in native nucleus pulposus cells (Fig. 7, Middle). There was an increase in sGAG content over the 21-day culture period, which was significant by day 21 (Fig. 7, Right). No significant difference in DNA content was observed over the 21-day culture period.

Discussion

This work demonstrates our ability to generate tissue-specific acellular matrices containing limited cell remnants and intact CSPGs for tissue engineering of the IVD. These matrices have no detectable remaining cytotoxic residuals and maintain fiber diameters similar to the native nucleus pulposus. Furthermore, the dNPs are able to be modified to create an injectable formulation that can support long-term culture of nucleus pulposus cells in vitro, suggesting it will be viable for in vivo studies.

Fig. 6. Scanning electron micrographs at varying magnifications from 5,000 $\times$ to 10,000 $\times$ revealed similar fibrillar structure before (A) and after (B) decellularization of the nuclei pulposi samples, as well as after solubilization (C). Average fiber diameters at 20,000 $\times$ of decellularized nuclei pulposi (dNPs) and solubilized dNPs were within 10% of the fresh nuclei pulposi controls (D).

An optimized decellularization process for nucleus pulposus needs to balance the removal of cells with the maintenance of sGAGs. The sGAGs are essential in maintaining the osmotic pressure of the nucleus pulposus [8], and they have the potential to play a role in driving cell phenotype [45–47,50,58] and preventing innervation [59–62]. The gentle decellularization protocol used in our laboratory for nerve [39,43,63] and lung tissue [38] was adapted for use in the nucleus pulposus by shortening the duration of wash cycles substantially. The use of amphoteric and anionic detergents in short cyclic intervals enabled the preservation of nucleus pulposus microstructure, while also maintaining 45% to 51% of sGAGs and removing >96% of DNA, similar to the methods previously developed by Mercuri et al. [44]. In addition, there were no detectable cytotoxic residuals after processing, as demonstrated by the elution study.

Preservation of sGAGs is an indirect indicator of preservation of CSPGs. In addition, our confocal results indicate preservation of CSPGs after decellularization. CSPGs are known to be potent neurite inhibitors [11,12,60,64]. By maintaining CSPG presence in the matrix, this has the potential to prevent nerve growth into the nucleus pulposus. Innervation of the nucleus pulposus has been demonstrated in patients with low back pain [13,14]. The ability of these solubilized dNP matrices to prevent nerve growth in vitro and in vivo is currently being explored.

One of the major outputs of this work is the creation of an in situ gelling decellularized nucleus pulposus. The location of the nucleus pulposus inside the annulus fibrosus makes it difficult to access without damaging the annulus. Injectable in situ gelling matrices are the most viable therapeutic option to prevent damage to the annulus fibrosus and future disc degeneration. Substantial work

Fig. 7. Viability of nucleus pulposus cell-seeded gels was high throughout the culture period (>89%) (Left). Nucleus pulposus cells were well spread and displayed morphology similar to cells in native tissue by day 21 (Middle) (calcein staining). Sulfated glycosaminoglycan content was significantly increased by day 21 (Right) ($p < .05$). Data ($n = 6$ per time point per batch) represent the mean $\pm$ standard error.

has been performed in other tissue types to create injectable in situ gelling decellularized matrices [37,51,54], but no work exists in the IVD. To date, the only injectable decellularized nucleus pulposus was developed by Illien-Jünger et al. [47]. However, this matrix was created by grinding the tissue to create small particles that were injected through a 25 gauge needle. This approach does not enable in situ gelation and formation of a material able to bear loads, and can potentially damage the anulus fibrosus. Ideally, a finer gauge needle would be used. Our work demonstrates the creation of an in situ gelling decellularized nucleus pulposus that has the ability to act as a cell carrier and be injected through a very fine needle (30 gauge) and still form a stable gel.

Matrix architecture and composition [65] can drive cell fate. Specifically, recent work has demonstrated that nucleus pulposus tissue alone has the potential to drive stem cells toward an NP phenotype [66,67]. This is likely due to the native microstructure as well as composition. Analysis via SEM revealed fiber diameters of dNP samples and injectable dNP samples were within 10% of fresh NP control samples. Furthermore, collagen and sGAG in the NP extracellular matrix were maintained during decellularization. Nucleus pulposus cell-seeded gels cultured over 21 days demonstrated high viability, NP-like morphology, and significantly increasing sGAG content over time, indicating an appropriate matrix environment. These data support the conclusion that the gel fiber morphology and composition of the samples are maintained during decellularization and solubilization, and have the potential to be ideal matrices for tissue engineering of the nucleus pulposus. Future work will explore the ability of the injectable dNPs created herein to drive both stem cells and induce pluripotent stem cells toward a nucleus pulposus cell phenotype.

Conclusions

In conclusion, we have created an acellular injectable tissue-specific matrix that is well tolerated in vitro by nucleus pulposus cells. This new matrix has the potential to be used for regenerative tissue engineering of the nucleus pulposus to restore disc height and prevent unwanted painful innervation.

References

- Andersson GB. Epidemiological features of chronic low-back pain. *Lancet* 1999;354:581-5.
- Pfarrmann CW, Metzendorf A, Zanetti M, Hodler J, Boos N. Magnetic resonance classification of lumbar intervertebral disc degeneration. *Spine* 2001;26:1873-8.
- Modic MT, Ross JS. Lumbar degenerative disk disease 1. *Radiology* 2007;245:43-61.
- Ross JS, Modic MT. Current assessment of spinal degenerative disease with magnetic resonance imaging. *Clin Orthop Relat Res* 1992;279:68-81.
- Modic M, Ross JS. Magnetic resonance imaging in the evaluation of low back pain. *Orthop Clin North Am* 1991;22:283-301.
- Modic M, Steinberg P, Ross J, Masaryk T, Carter J. Degenerative disk disease: assessment of changes in vertebral body marrow with MR imaging. *Radiology* 1988;166:193-9.
- Wang Y, Battié MC. Epidemiology of lumbar disc degeneration. The intervertebral disc. Vienna: Springer; 2014. p. 139-56.
- Shapiro IM, Risbud MV. Introduction to the structure, function, and comparative anatomy of the vertebrae and the intervertebral disc. The intervertebral disc. Springer; 2014. p. 3-15.
- Melrose J, Roughley P. Proteoglycans of the intervertebral disc. The intervertebral disc. Springer; 2014. p. 53-77.
- Richardson SM, Freemont AJ, Hoyland JA. Pathogenesis of intervertebral disc degeneration. The intervertebral disc. Vienna: Springer; 2014. p. 177-200.
- Silver J, Miller JH. Regeneration beyond the glial scar. *Nat Rev Neurosci* 2004;5:146-56.
- Jones LL, Sajed D, Tuszynski MH. Axonal regeneration through regions of chondroitin sulfate proteoglycan deposition after spinal cord injury: a balance of permissiveness and inhibition. *J Neurosci* 2003;23:9276-88.
- García-Cosamalón J, Del Valle ME, Calavia MG, et al. Intervertebral disc, sensory nerves and neurotrophins: who is who in nociceptive pain? *J Anat* 2010;217:1-15.
- Ozawa T, Ohtori S, Inoue G, Aoki Y, Moriya H, Takahashi K. The degenerated lumbar intervertebral disc is innervated primarily by peptide-containing sensory nerve fibers in humans. *Spine* 2006;31:2418-22.
- Vo NV, Hartman RA, Yurube T, Jacobs LJ, Sowa GA, Kang JD. Expression and regulation of metalloproteinases and their inhibitors in intervertebral disc aging and degeneration. *Spine J* 2013;23:331-41.
- Sobajima S, Shimer AL, Chadderton RC, et al. Quantitative analysis of gene expression in a rabbit model of intervertebral disc degeneration by real-time polymerase chain reaction. *Spine J* 2005;15:14-23.
- Wang S, Rui Y, Lu J, Wang C. Cell and molecular biology of intervertebral disc degeneration: current understanding and implications for potential therapeutic strategies. *Cell Prolif* 2014;47:381-90.
- Yang X, Li X. Nucleus pulposus tissue engineering: a brief review. *Eur Spine J* 2009;18:1564-72.
- Crevensten G, Walsh AJ, Ananthakrishnan D, et al. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. *Ann Biomed Eng* 2004;32:430-4.
- Masuda K. Biological repair of the degenerated intervertebral disc by the injection of growth factors. *Eur Spine J* 2008;17:441-51.
- Joshi A, Fussell G, Thomas J, et al. Functional compressive mechanics of a PVA/PVP nucleus pulposus replacement. *Biomaterials* 2006;27:176-84.
- Revell P, Damien E, Di Silvio L, Gurav N, Longinotti C, Ambrosio L. Tissue engineered intervertebral disc repair in the pig using injectable polymers. *J Mater Sci Mater Med* 2007;18:303-8.
- Allen MJ, Schoonmaker JE, Bauer TW, Williams PF, Higham PA, Yuan HA. Preclinical evaluation of a poly (vinyl alcohol) hydrogel implant as a replacement for the nucleus pulposus. *Spine* 2004;29:515-23.
- Cloyd JM, Malhotra NR, Weng L, Chen W, Mauck RL, Elliott DM. Material properties in unconfined compression of human nucleus pulposus, injectable hyaluronic acid-based hydrogels and tissue engineering scaffolds. *Eur Spine J* 2007;16:1892-8.
- Gloria A, Borzacchiello A, Causa F, Ambrosio L. Rheological characterization of hyaluronic acid derivatives as injectable materials toward nucleus pulposus regeneration. *J Biomater Appl* 2012;26:745-59.
- Peroglio M, Grad S, Mortisen D, et al. Injectable thermoreversible hyaluronan-based hydrogels for nucleus pulposus cell

- encapsulation. *Eur Spine J* 2012;21:839-49.
27. Bertolo A, Mehr M, Aebli N, Baur M, Ferguson SJ, Stoyanov JV. Influence of different commercial scaffolds on the in vitro differentiation of human mesenchymal stem cells to nucleus pulposus-like cells. *Eur Spine J* 2012;21:826-38.
 28. Narayanan G, Vernekar VN, Kuyinu EL, Laurencin CT. Poly (lactic acid)-based biomaterials for orthopaedic regenerative engineering. *Adv Drug Deliv Rev* 2016;doi:10.1016/j.addr.2016.04.015.
 29. Damodaran VB, Bhatnagar D, Murthy NS. Biomedical polymers: an overview. *Biomedical polymers*. Springer; 2016. p. 1-22.
 30. Chua LS, Kim H-W, Lee JH. Signaling of extracellular matrices for tissue regeneration and therapeutics. *Tissue Eng Regen Med* 2016;13:1-12.
 31. Li S-T. Tissue-derived biomaterials (collagen). The biomedical engineering handbook. Boca Raton, FL.: CRC Press; 1995. p. 627-47.
 32. Kim B-S, Baez CE, Atala A. Biomaterials for tissue engineering. *World J Urol* 2000;18:2-9.
 33. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. *Biomaterials* 2011;32:3233-43.
 34. Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: structure and function. *Acta Biomater* 2009;5:1-13.
 35. Gilbert TW. Strategies for tissue and organ decellularization. *J Cell Biochem* 2012;113:2217-22.
 36. Gilbert TW, Sellaro TL, Badylak SF. Decellularization of tissues and organs. *Biomaterials* 2006;27:3675-83.
 37. Singelyn JM, DeQuach JA, Seif-Naraghi SB, Littlefield RB, Schup-Magoffin PJ, Christman KL. Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. *Biomaterials* 2009;30:5409-16.
 38. Nagao RJ, Ouyang Y, Keller R, Lee C, Suggs LJ, Schmidt CE. Preservation of capillary-beds in rat lung tissue using optimized chemical decellularization. *J Mater Chem B* 2013;1:4801-8.
 39. Hudson TW, Liu SY, Schmidt CE. Engineering an improved acellular nerve graft via optimized chemical processing. *Tissue Eng* 2004;10:1346-58.
 40. Reing JE, Brown BN, Daly KA, et al. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. *Biomaterials* 2010;31:8626-33.
 41. DeQuach JA, Yuan SH, Goldstein LS, Christman KL. Decellularized porcine brain matrix for cell culture and tissue engineering scaffolds. *Tissue Eng A* 2011;17:2583-92.
 42. Crapo PM, Medberry CJ, Reing JE, et al. Biologic scaffolds composed of central nervous system extracellular matrix. *Biomaterials* 2012; 33:3539-47.
 43. Hudson TW, Zawko S, Deister C, et al. Optimized acellular nerve graft is immunologically tolerated and supports regeneration. *Tissue Eng* 2004;10:1641-51.
 44. Mercuri JJ, Gill SS, Simionescu DT. Novel tissue-derived biomimetic scaffold for regenerating the human nucleus pulposus. *J Biomed Mater Res A* 2011;96:422-35.
 45. Mercuri JJ, Patnaik S, Dion G, Gill SS, Liao J, Simionescu DT. Regenerative potential of decellularized porcine nucleus pulposus hydrogel scaffolds: stem cell differentiation, matrix remodeling, and biocompatibility studies. *Tissue Eng A* 2013;19:952-66.
 46. Yuan M, Yeung CW, Li YY, et al. Effects of nucleus pulposus cell-derived acellular matrix on the differentiation of mesenchymal stem cells. *Biomaterials* 2013;34:3948-61.
 47. Illien-Jünger S, Sedaghatpour DD, Laudier DM, Hecht AC, Qureshi SA, Iatridis JC. Development of a bovine decellularized extracellular matrix-biomaterial for nucleus pulposus regeneration. *J Orthop Res* 2016;34:876-88.
 48. Xu H, Xu B, Yang Q, et al. Comparison of decellularization protocols for preparing a decellularized porcine annulus fibrosus scaffold. *PLoS ONE* 2014;9:e86723.
 49. Wu L-C, Chiang C-J, Liu Z-H, Tsuang Y-H, Sun J-S, Huang Y-Y. Fabrication and properties of acellular porcine annulus fibrosus for tissue engineering in spine surgery. *J Orthop Surg* 2014;9:1.
 50. Chan LK, Leung VY, Tam V, Lu WW, Sze K, Cheung KM. Decellularized bovine intervertebral disc as a natural scaffold for xenogenic cell studies. *Acta Biomater* 2013;9:5262-72.
 51. Freytes DO, Martin J, Velankar SS, Lee AS, Badylak SF. Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. *Biomaterials* 2008;29:1630-7.
 52. Kwon JS, Yoon SM, Shim SW, et al. Injectable extracellular matrix hydrogel developed using porcine articular cartilage. *Int J Pharm* 2013;454:183-91.
 53. Lee JS, Shin J, Park H-M, et al. Liver extracellular matrix providing dual functions of two-dimensional substrate coating and three-dimensional injectable hydrogel platform for liver tissue engineering. *Biomacromolecules* 2013;15:206-18.
 54. Farnebo S, Woon CY, Schmitt T, et al. Design and characterization of an injectable tendon hydrogel: a novel scaffold for guided tissue regeneration in the musculoskeletal system. *Tissue Eng A* 2014; 20:1550-61.
 55. Zhao L, Weir MD, Xu HH. An injectable calcium phosphate-alginate hydrogel-umbilical cord mesenchymal stem cell paste for bone tissue engineering. *Biomaterials* 2010;31:6502-10.
 56. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671-5.
 57. Schindelin J, Rueden CT, Hiner MC, Eliceiri KW. The ImageJ ecosystem: an open platform for biomedical image analysis. *Mol Reprod Dev* 2015;82:518-29.
 58. Mwale F, Roughley P, Antoniou J. Distinction between the extracellular matrix of the nucleus pulposus and hyaline cartilage: a requisite for tissue engineering of intervertebral disc. *Eur Cell Mater* 2004;8:63-4.
 59. Wang H, Katagiri Y, McCann TE, et al. Chondroitin-4-sulfation negatively regulates axonal guidance and growth. *J Cell Sci* 2008;121:3083-91.
 60. Gilbert RJ, McKeon RJ, Darr A, Calabro A, Hascall VC, Bellamkonda RV. CS-4, 6 is differentially upregulated in glial scar and is a potent inhibitor of neurite extension. *Mol Cell Neurosci* 2005;29:545-58.
 61. Brown JM, Xia J, Zhuang B, et al. A sulfated carbohydrate epitope inhibits axon regeneration after injury. *Proc Natl Acad Sci* 2012; 109:4768-73.
 62. Purmessur D, Cornejo MC, Cho SK, et al. Intact glycosaminoglycans from intervertebral disc-derived notochordal cell-conditioned media inhibit neurite growth while maintaining neuronal cell viability. *Spine J* 2015;15:1060-9.
 63. Nagao RJ, Lundy S, Khaing ZZ, Schmidt CE. Functional characterization of optimized acellular peripheral nerve graft in a rat sciatic nerve injury model. *Neurol Res* 2011;33:600-8.
 64. Tom VJ, Steinmetz MP, Miller JH, Doller CM, Silver J. Studies on the development and behavior of the dystrophic growth cone, the hallmark of regeneration failure, in an in vitro model of the glial scar and after spinal cord injury. *J Neurosci* 2004;24:6531-9.
 65. Zhou X, Tao Y, Wang J, et al. Three-dimensional scaffold of type II collagen promote the differentiation of adipose-derived stem cells into a nucleus pulposus-like phenotype. *J Biomed Mater Res A* 2016;104:1687-93.
 66. Liu Y, Rahaman MN, Bal BS. Modulating notochordal differentiation of human induced pluripotent stem cells using natural nucleus pulposus tissue matrix. *PLoS ONE* 2014;9:e100885.
 67. Liu Y, Fu S, Rahaman MN, Mao JJ, Bal BS. Native nucleus pulposus tissue matrix promotes notochordal differentiation of human induced pluripotent stem cells with potential for treating intervertebral disc degeneration. *J Biomed Mater Res A* 2015;103:1053-9.