Accelerated chondrogenesis in nanofiber polymeric scaffolds embedded with BMP-2 genetically engineered chondrocytes

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ABSTRACT

This study evaluated chondrogenesis within a nanofiber polymeric scaffold seeded with isolated untreated chondrocytes, isolated chondrocytes genetically engineered with adenoviral (Ad) bone morphogenetic protein (BMP)-2, or isolated chondrocytes genetically engineered with green fluorescent protein (Ad-GFP). Electrospun polycaprolactone scaffolds (150-200 μm thickness, 700 μm fiber diameter, 30 μm pore size) were optimally seeded with 1 x 10^7 isolated chondrocytes by using a 20% serum gradient culture system. Chondrocyte-scaffold constructs (untreated, Ad-BMP-2 and Ad-GFP) were generated from 5 adult horses, cultured in triplicate for 7 or 14 days, and quantitatively analyzed for cell proliferation (DNA content; Hoechst assay), viability, morphology (confocal microscopy), matrix production (proteoglycan content; DMMB assay), and mRNA expression of collagen I, collagen II, and aggrecan. Chondrocytes transduced with Ad-BMP-2 demonstrated greater cell numbers and significantly greater expression of chondrogenic markers including aggrecan, collagen II, and proteoglycan through 14 days of culture as compared to untransduced or Ad-GFP controls. This study demonstrated that chondrocytes can be driven to seed a polycaprolactone nanofiber scaffold by serum gradient and a polycaprolactone nanofiber scaffold containing Ad-BMP2 transduced chondrocytes resulted in greater and accelerated chondrogenesis than controls. This cell engineered construct has potential use in one-step cartilage repair in vivo.

Keywords: Nanofiber; Scaffold; Chondrogenesis; BMP-2; Adenovirus

1. INTRODUCTION

Articular cartilage defects heal poorly and combinations of progenitor chondrocytes, bioactive factors, and matrices are being applied as focal synthetic devices [1-3]. Synthetic biodegradable polymers have served as scaffolds for articular cartilage tissue engineering. Studies have demonstrated that properties such as pore size, material composition, and diameter of the fibers are important for cell proliferation, adhesion, migration and maintenance of a chondrogenic phenotype of seeded cells [4-12]. Successful biodegradable polymeric scaffolds permissive to cell and tissue in-growth have been studied both in vitro and in vivo, including for chondrocytes [4,6-13]. Properties of successful scaffolds include interconnected pores permissive to cell adhesion, proliferation and migration, and fluid transport as well as biocompatibility. Scaffolds have been created from bioactive materials such as plasma, starch, collagen, hydrogel, hydroxyapatite, alginate, and periosteum [9,11,13-15] as well as the synthetic materials such as porous poly (l-lactic acid) [5,8,16], poly (glycerol sebacate) (PGS)[6], poly (1,8 octanediol- co-citrate) (POC) [6], poly (ethyleneglycol)-terephtha- late/poly (butylene terephthalate (PEGT/PBT) [17] poly-caprolactone (PCL) [4-7,10,12,17-23] and poly (gamma- glutamic acid)-graft-chondroitin sulfate/PCL composites [24], polydioxone [25] and other PCL composites [8,13].

Recent publications support the use of a nanoscale fiber size and PCL material for tissue engineering of chondrocytes [4-6,9-10,12,21,23]. Use of a fiber material in a mesh pattern has been reported for chondrocytes and the optimum fiber material will consider fiber size and topology. Nanofiber technology and its influence on cell behavior is advancing the science of tissue engineering by providing a fiber on the scale of biologic fibers [18-20,23]. It is proposed that fibers which closely mimic...
normal extra-cellular matrix or basement membrane structure will show improved cytocompatibility. Typically, naturally occurring extra-cellular matrices and basement membranes are composed of proteins including fibronectin, collagen, hyaluronic acid, chondroitin sulfate, dermatan sulfate and proteoglycans. These proteins are fibrous and are on a nanofiber scale in size. PCL fibers in the 30-1500 nm diameter range have been demonstrated to have optimal structural integrity and, particularly under dynamic loading, supported a desirable cellular response in culture, including chondrocyte proliferation and matrix production [23].

Electrospinning is a well-established process that can produce a random meshwork of nanofibers, including PCL, with appropriate pore size to support cellular infiltration of the scaffold, including chondrocytes [4-5,9-10,12,23-24], stem cells [21,22,26], glioma cells [27], and endothelial cells [28].

Cell-based therapy in conjunction with scaffolds for tissue engineering of cartilage is supported in vitro [3,6,8,10-12,23,24,26] and in vivo [7,14,17:21-22,25] and is rapidly advancing toward clinical application [1-4]. Typically the cell source for cartilage engineering is chondrocytes [3,6-12,14,21,23-25] or mesenchymal stem cells. [17,21-22,26,29-30] Specifically for articular cartilage repair, animal studies support the use of biodegradable scaffolds seeded with morcelized cartilage [25], chondrocytes [7,14,21] or direct injection of mesenchymal stem cells [31]. Supplementation with growth factors, such as transforming growth factor-beta 3, a known regulator of cell growth and differentiation, could enhance chondrocyte density and integration [11]. Growth factor members of the TGF-beta superfamily, such as the BMPs, are particularly beneficial in promoting chondrogenesis of mesenchymal stem cells [29-34] and can support cartilage matrix production [35,36]. Specifically, bone morphogenetic protein (BMP)-2 can regulate chondrocyte differentiation in progenitor cells [34], enhance bone formation through the endochondral ossification pathway [31,37], and can increase chondrocyte extracellular matrix production in vitro [35-36]. Mesenchymal stem cells genetically engineered to produce BMP-2 can enhance articular cartilage repair during articular fracture repair in vivo [31]. Our study focuses on the ability of the BMP-2 gene to support phenotype, proliferation, and matrix production of chondrocytes suspended in a biodegradable nanofiber PCL scaffold. We investigated whether BMP-2 could successfully promote chondrogenesis in this 3-dimensional scaffold for potential use in articular cartilage tissue engineering.

2. METHODS

2.1. Study Design

Chondrocytes were isolated and expanded in primary monolayer culture. Chondrocytes were seeded onto the surface of three dimensional polycaprolactone nanofiber scaffolds and evaluated for scaffold penetration by histology, crossection and morphology by confocal microscopy under conditions of fetal bovine serum gradient (10 or 30%), cell seeding density (5.0 × 10^5/ml, 1.0 × 10^6 ml, or 5.0 × 10^6 ml) and duration of cell growth (day 2,7, or 14). The best condition was selected and chondrocytes from 5 horses were seeded onto similar scaffolds, in duplicate, and were evaluated at two time points (day 7 or 14) for three cell preparations; isolated cells (untreated control), isolated cells transduced with Ad-GFP (vector control), and isolated cells transduced with Ad human (h) BMP-2 (experimental gene). Chondrocyte transduction and BMP-2 production were confirmed. Outcome assessments were cell proliferation, cell morphology, cell viability, BMP-2 production, extracellular proteoglycan matrix production, and chondrocyte gene expression of Type I and Type II collagen as well as aggrecan.

2.2. Generation of Adenoviral Vector Constructs

Recombinant adenoviral vector containing these 1547 base-pairs of human BMP-2 under the cytomegalovirus promoter were propagated [31]. Expression of transgene was verified in cell culture.

2.3. Chondrocyte Preparation

Chondrocytes from 5 adult horses [5-9 years] were harvested aseptically from articular cartilage of the femoropatellar joint and isolated by collagenase digestion. Chondrocytes were expanded in monolayer in Dulbeco’s Modified Eagle Medium (DMEM; Gibco, Sigma-Aldrich, St. Louis, MO) supplemented with sodium penicillin at a concentration of 50 units/ml, streptomycin at a concentration of 100 units/ml, and L-glutamine at a concentration of 29.2 mg/ml (supplemented DMEM) with 10% fetal bovine serum (FBS). At 75% confluence, cells were lifted, counted and pooled in equal concentrations and cultured in monolayer in flasks. Chondrocyte monolayer flasks had Ad vector transduction with Ad-GFP or Ad-BMP-2 performed at a multiplicity of infection (moi) of 17:1 (Adeno-X™ Rapid Titer Kit, BD Biosciences Clontech, Palo Alto, CA) at 37°C for a transduction time of 2 h, washed and allowed to incubate overnight to achieve expression of transgene product. Transduction efficiency was determined by calculating the percent of cells fluorescing [525 nm wavelength] per microscopic field (200X) in the monolayer chondrocytes treated with Ad-GFP. Chondrocytes were harvested and allocated to PCL culture systems.

2.4. Polycaprolactone Scaffolds

Nanofibrous (~700 nm diameter) PCL [MW 40,000,
Sigma Aldrich, St. Louis, MO] matrices were created through an electrospinning process[18-22] to form 150-200 μm thick 3 dimensional sheets containing ~88% porosity and an average pore size of 30μm.[18] Sheets were cut into 25 mm diameter discs using a 25 mm circular leather punch and sterilized by ethanol treatment.

2.5. Chondrocyte Culture on PCL Matrices

The 25 mm diameter sterile 3D nano-fibrous PCL matrices were placed into Corning Costar Snapwell 12 mm inserts™ [Corning Inc., Corning, NY] designed to fit into standard 6-well culture plates. Chondrocytes were seeded onto the PCL scaffolds by placing cells in supplemented DMEM into the Snapwell® inserts. Inserts were placed into standard 6-well culture plates containing supplemented DMEM with the assigned concentration of FBS to create a gradient of FBS across the scaffold construct.

2.6. Preliminary Study

Isolated chondrocytes were cultured on PCL matrices in triplicate for 18 different conditions representing 3 cell seeding densities of 5.0 × 10⁵/ml, 1.0 × 10⁶/ml, or 5.0 × 10⁶/ml, 2 serum gradients of 10% or 30%, and duration of culture of 2, 7 and 14 days. PCL/ cell matrices were embedded in OCT medium, snap frozen in liquid nitrogen, cryosectioned (10 microns), and stained with toluidine blue for microscopy or fixed for surface scanning electron microscopy (SEM) to assess fiber pattern and cell distribution. The cell seeding density and serum gradient that supported the greatest number and depth of penetration of chondrocytes in the PCL matrix was selected for use in subsequent experiments.

2.7. Chondrogenesis of Genetically Engineered PCL/ Cell Matrices

Using the best seeding conditions, isolated chondrocytes from 5 horses were cultured on PCL matrices in 12 replicates each of untreated, transduced with Ad-GFP, or transduced with Ad-hBMP-2 for 14 days. Media were changed on days 2, 7, and 14 and stored at -80°C. Constructs from at least 2 replicates from each horse from each treatment (untreated, Ad-GFP-treated and Ad-BMP-2-treated) cultured for 7 or 14 days were quantitatively evaluated for each parameter of cell proliferation (DNA [μg/ml]; Hoechst assay), % viability (live/ dead stain) and morphology [confocal microscopy], matrix proteoglycan expression (ng/ml; DMBB assay) and gene expression (mRNA quantitative RT-PCR) of aggrecan (ddCT), collagen I (ddCT) and collagen II (ddCT) using equine specific primers and probes. Aggrecan and collagen gene expression intensity was expressed as a ration to 18sRNA and between Ad-BMP-2 treated chondrocytes to untreated and Ad-GFP controls (ddCT). BMP-2 protein concentration (ng/ml; ELISA) in the media and chondrocyte GFP expression intensity were compared among untreated, Ad-GFP-treated and Ad-BMP-2-treated PCL/ cell matrices.

2.8. Transgene and Protein Expression

GFP fluorescence was quantified using an in vivo imaging system (IVIS®, Xenogen Corporation, Alameda, CA) at day 2 post- transduction. GFP production was quantified as flux (photons of light produced per second per square centimeter per steradian, photons/s/cm²/sr).[31]

Aliquots of media from PCL/ cell matrix culture systems were frozen at -80°C on days 2, 7, and 14. Production of hBMP-2 was quantified using enzyme-linked immunosobent assays (ELISAs) for recombinant human (rh) BMP-2 (Quantikine®, R & D Systems, Minneapolis, MN) and expressed as picograms/millilitre/day.

2.9. Cytomorphology of PCL/ Cell Matrices

Cell morphology and viability were quantified using special stains [LIVE/DEAD® Viability Kit, Molecular Probes Inc. OR] and confocal microscopy [Leica DM-IRE2, Leica Microsystems Inc., Bannockburn, IL]. Cell morphology was scored after staining the cytoskeleton (actin) with phalloxitin (Alexa Fluoro 647 Phalloidin, Molecular Cell Probes Inc. Oregon) and the nucleus with DAPI (Molecular Probes Inc. OR). Three representative fields were scored 0-4 with 0 representing the most healthy adherent cell and 4 representing the most pyknotic and crenated cell.

2.10. Cellular Content

Cellular content was determined by analyzing DNA content of PCL/ cell matrices using a modification of the previously described Hoechst 33258 Fluorometric assay. Discs of PCL/ matrices containing chondrocytes cultured for 7 or 14 days were placed into a 2.0 ml Eppendorf tube with 1 ml of papain (Acros Organics) dissolved at 125 μg/ml in sterile 1X PBS pH 6.0, with 5mM cysteine HCl and 5mM Na²EDTA and incubated for 24h at 60°C. Papain digested samples were centrifuged at 1500 rpm (0.2 rcf) for 30 seconds to pellet debris and the supernatant isolated. DNA standards of double stranded calf thymus DNA (Sigma) dissolved in TN buffer (50 mM Tris pH 7.5, 150 mM NaCl) and serially diluted ranged from 10 μg/ml to 500 μg/ml. in a Costar® 96 well, black, clear bottom assay plate (Corning, Inc.) 50μl of papain digested sample or standard was placed in 200μl of Hoechst 33258 dye (AnaSpec, Inc) diluted to 0.2 μg/ml in TN buffer. The plates were read on a UV/Vis spectrometer (Lambda 45, Perkin Elmer) at an excitation of 360 nm, and emission of 460 nm, with a 430 nm cutoff filter. DNA concentrations of samples were determined
from the calf thymus DNA standard curve and reported in μg/ml.

2.11. Proteoglycan Production in PCL/Cell Matrices

Extracellular production of proteoglycan was determined using a dimethylmethylene blue assay (DMMB assay) on papain digested supernatant from samples. DMMB reagent was prepared by adding 16mg 1, 9 dimethylmethylen blue (Polysciences, Inc) to 5ml ethanol followed by the addition of 2 ml of formic acid and 2g sodium formate. The volume was then brought to 1000 ml with distilled water. Chondroitin Sulfate A from bovine trachea (Calbiochem) was serially diluted to create standards between 5 ng/ml and 50 ng/ml. The assay was performed by combining 50 μl of standard solution or sample solution with 200 μl of the DMMB staining reagent and immediately determining the absorbance at 550 nm with a BioMate 3 Spectrophotometer (Thermo Electron Corporation). Proteoglycan concentrations were determined from the Chondroitin Sulfate standard curve and reported in ng/ml.

2.12. Chondrocyte Gene Expression

Discs of PCL/Chondrocyte matrices were collected at day 7 and 14 of culture and placed into a 2.0 ml Eppendorf tube with 1 ml of a commercially available reagent composed of a monophasic solution of phenol and guanidine isothiocyanate (Trizol® reagent, GibcoBRL, Life Technologies, Frederick, MD). The PCL completely dissolved in the Trizol® reagent and total mRNA extracted with guanidine thiocyanate/phenol/chloroform and stored at -80°C for real time reverse transcription polymerase chain reaction (RT-PCR) analysis. Equine specific primers and probes were designed and used (Primer Express software, Applied Biosystems Inc., Foster City, CA) to amplify and detect equine aggrecan, equine collagen type Ia, and equine collagen type II mRNA (Table 1). Relative gene expression was quantified by RT-PCR using 18s ribosomal RNA for normalization (Taqman 7000 sequence Detection System, Applied Biosystems Inc.). All Taqman probes were labeled at the 5’ end with 6-carboxy-fluorescene (FAM) and at the 3’ end with a minor groove binder non-fluorescent quencher. The thermal cycle protocol was 50°C for 2 in, 60°C for 30 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

2.13. Statistical Analysis

Quantitative data (aggrecan [ddCT], collagen I [ddCT], collagen II [ddCT], proteoglycan [ng/ml], % chondrocyte viability, BMP-2 concentration [pg/ml]) were analyzed for difference among the three groups across days using a repeated two-factor analysis of variance with statistical significance set at P < 0.05. Scored data (morphology) was analyzed among groups with a Kruskal-Wallis Rank test (P < 0.05).

3. RESULTS

3.1. Confirmation of Gene Transduction: GFP

Transduction efficiency (GFP positive cells) was > 80% and only detected in Ad-GFP transduced chondrocytes. GFP expression intensity was high and sustained for the 14 days of the study (Figure 1).

Table 1. Equus caballus primer and probe sequences for RT-PCR analysis of gene sequences.

<table>
<thead>
<tr>
<th>Type</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tr>
<td>Type II collagen</td>
<td>5'-AAGAGCCGGAGACTGGATTGAC-3'</td>
<td>5'-TCCATGTGTCAGAAGACCTCA-3'</td>
<td>5'-AACCAAGGGCTGCAACCTTAGAGGCC-3'</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>5'-CCGTTCTTCTTGTGACGATG-3'</td>
<td>5'-CCGTTCAATTGAGAGGCTCA-3'</td>
<td>5'-ACCTGAAAACGATAACCAT-3'</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>5'-CGAGTTGCAGATGCTGACACACT-3'</td>
<td>5'-CGGCTGGTCAGATGCTGACACACT-3'</td>
<td>5'-CTTGCAATTTGAGAAGTTTCGCCC-3'</td>
</tr>
</tbody>
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Figure 1. IVIS analysis of positive GFP fluorescence in Ad-GFP transduced chondrocytes (central 3 wells). Adjacent wells contained untransduced cells and Ad-BMP-2 transduced cells which had no GFP expression.
3.2. Transgene Protein Expression

Gene transduction and protein expression were confirmed for the Ad-BMP-2-transduced chondrocytes in the PCL matrices on days 7 and 14 of culture. BMP concentration was > 150,000 pg/ml in Ad-BMP-2-transduced PCL/ cell matrices and <100 pg/ml in untreated and Ad-GFP PCL/ cell matrices.

3.3. Scanning Electron Microscopy

Scanning electron microscopy of PCL scaffolds demonstrated a random woven pattern of fibers in the 300-1000 nm range with an average pore size of ~30 nm (Figure 2). Toluidine blue stained frozen cross sections of the PCL/ cell scaffolds demonstrated penetration to ¾ depth by 5 × 105 and 1 × 106 cells/ scaffold in a 30% serum gradient by day 7 (Figures 3A-B). Cell seeding density of 1 × 105 cells, 10% serum gradient and 2 days were insufficient to produce chondrocyte penetration of PCL matrices. Confocal microscopy of stained untreated chondrocytes and GFP expressing chondrocytes seeded on PCL matrices demonstrated an even distribution of cells with normal morphology, sustained gene expression, and cell viability in situ for 14 days (Figures 4A-B).

3.4. Cytomorphology of PCL/ Cell Matrices

Cell morphology score (median 4; range 3-4) and nuclear morphology score (median 3; range 3-4) was not different (P > 0.05) among untreated, Ad-GFP, and Ad-BMP-2 PCL/ cell matrices at both days 7 and 14.

3.5. Cell Content

DNA content (µg/ml) was not different among untreated, Ad-GFP, and Ad-BMP-2 PCL/ cell matrices at day 7, but was significantly decreased in Ad-GFP and increased in Ad-BMP-2 by day 14 (P < 0.01) (Figure 5A).

3.6. Proteoglycan Production in PCL/ Cell Matrices

Proteoglycan content (ng/ml) within the PCL/ cell matrices was significantly greater in the Ad-BMP-2 treated matrices by day 7 and continued to significantly increase only in the Ad-BMP-2 matrices (P < 0.05) (Figure 5B).

3.7. Chondrocyte Gene Expression

Quantitative gene expression was expressed as the inverse of Delta CT values so that positive values correlated to increased gene expression. Ad-BMP-2 matrices has significantly greater Type II collagen (P < 0.002) and aggrecan (P < 0.001) gene expression than untreated and Ad-GFP matrices by day 7 and sustained until at least day 14. There was no difference in type I collagen gene expression among groups (Figures 6A-C).
DNA content (a) decreased on day 14 as compared to day 7 except in the Ad-BMP2 treated group (p < 0.01) which sustained their cell numbers. Cells transduced with Ad-BMP2 (b) had significantly greater proteoglycan content by day 7 (p < 0.05), which had further increased by day 14 (p < 0.03).

Cells transduced with Ad-BMP2 had significantly greater gene expression of chondrogenic markers including Type II collagen (a) and aggrecan (b) and significantly less gene expression of Type I collagen (c).

4. DISCUSSION

Our data demonstrated that a serum gradient can be used to attract chondrocytes to seed into an electrospun PCL nanofiber scaffold and sustain chondrogenic phenotype in standard media. Nanofiber scaffolds offer the advantage of smaller biologic fiber diameters, but this woven mesh can be a challenge for cells to permeate the scaffold. Our work demonstrated this as the chondrocytes remain-
ed on the surface in the control specimens. This is particularly challenging for relatively nonmobile chondrocytes that must dedifferentiate to a fibroblastic phenotype to migrate along fibrils and into pores. In our study, BMP2 sustained chondrogenic phenotype and extracellular matrix production while permitting these cells to enter the scaffold. For cartilage engineering, migration is particularly relevant to accommodate the thickness of a scaffold desired for cartilage regeneration (100-300 uM). Although it is possible to incorporate cells into a scaffold using an in situ cell seeding protocol [38], or by modifying the electrospinning protocol to produce larger fibers (in the micrometer range with larger pore sizes of > 100 uM [23,29]), these methods are potentially handicapped by injury or contamination of cells or loss of the bioactivity advantage of the nanofibrous scale. In vivo generation of a serum gradient in the base of a cartilage defect is potentially achievable with the use of autologous serum products placed in the bed of debrided cartilage defects.[40] Commercially available platelet rich plasma or plasma concentrate products have been shown to have increased growth factor concentration of TGFbeta1 and 2 as well as other growth factors that may serve as a chemoattractant to chondrocytes layered on the surface of a scaffold in vivo. [41] Studies to further investigate this are warranted. These biologic plasma/fibrin products offer the additional advantage of serving as a biologic glue that may be able to help secure the scaffold into the defect. Fibrin glue is reported to secure cells within full-thickness cartilage defects in animal models [42].

In our study, the chondrocytes engineered to express BMP-2 had accelerated and amplified chondrogenesis within the scaffold as compared to control cells. In a previous in vitro study, chondrocytes incorporated in a hydrogel had enhanced matrix generation when the media was supplemented with TGFbeta3, a known chondrogenic growth factor. [11] Supplementing cells within a scaffold with a growth factor solution is technically difficult in vivo, other than by using a serum/plasma product as described above. Saturation of the scaffold with a TGFbeta solution is likely to have the TGFbeta rapidly leave the site or be diluted by joint fluid. Engineering of chondrocytes with chondrogenic genes offers an alternative for sustained trophic influences on the cells within the scaffold as demonstrated with BMP2 in our study. Additionally the release of soluble BMP2 will have a paracrine effect on other cells migrating into the site to heal the defect such as bone marrow-derived cells or synovial fibroblasts. Other genes such as insulin-like growth factor or TGFbeta may function similarly and could be used in chondrocyte engineering. The process of chondrocyte transduction with adenovirus did not nullify this trophic effect in our study. Methods of cell transduction other than use of adenovirus may offer advantages, most notably a more sustained gene expression [43]. Our data provided evidence that engineering cells can provide enhanced chondrogenesis in Electrospun PCL nanofiber scaffolds for use in cartilage tissue engineering.

Current techniques under investigation for autologous chondrocyte transplantation include direct intraoperative processing of autologous articular cartilage to expose or isolate chondrocytes for immediate reimplantation [25] or chondrocyte expansion in culture prior to reimplantation at a second surgery. Gene transduction with BMP-2 can occur in under 2 hours and could be a practical genetic engineering technique to augment autologous cells with genes promoting chondrogenesis in the operating room. Other techniques that focus on autologous chondrocyte expansion prior to reimplantation at a second surgery would also be readily amenable to genetic engineering as described in this report. This study provides evidence that this process holds merit for potential clinical application. Our study was limited to in vitro processing and further studies to confirm this potential in an in vivo animal model are warranted.

REFERENCES

modification of electrospun polycaprolactone nanofiber meshes by plasma treatment to enhance biological performance. Small, 5(10), 1195-1206.


