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Fabrication of anatomically-shaped cartilage constructs using decellularized cartilage-derived matrix scaffolds

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ABSTRACT

The native extracellular matrix of cartilage contains entrapped growth factors as well as tissue-specific epitopes for cell-matrix interactions, which make it a potentially attractive biomaterial for cartilage tissue engineering. A limitation to this approach is that the native cartilage extracellular matrix possesses a pore size of only a few nanometers, which inhibits cellular infiltration. Efforts to increase the pore size of cartilage-derived matrix (CDM) scaffolds dramatically attenuate their mechanical properties, which makes them susceptible to cell-mediated contraction. In previous studies, we have demonstrated that collagen crosslinking techniques are capable of preventing cell-mediated contraction in CDM disks. In the current study, we investigated the effects of CDM concentration and pore architecture on the ability of CDM scaffolds to resist cell-mediated contraction. Increasing CDM concentration significantly increased scaffold mechanical properties, which played an important role in preventing contraction, and only the highest CDM concentration (11% w/w) was able to retain the original scaffold dimensions. However, the increase in CDM concentration led to a concomitant decrease in porosity and pore size. Generating a temperature gradient during the freezing process resulted in unidirectional freezing, which aligned the formation of ice crystals during the freezing process and in turn produced aligned pores in CDM scaffolds. These aligned pores increased the pore size of CDM scaffolds at all CDM concentrations, and greatly facilitated infiltration by mesenchymal stem cells (MSCs). These methods were used to fabricate of anatomically-relevant CDM hemispheres. CDM hemispheres with aligned pores supported uniform MSC infiltration and matrix deposition. Furthermore, these CDM hemispheres retained their original architecture and did not contract, warp, curl, or splay throughout the entire 28-day culture period. These findings demonstrate that given the appropriate fabrication parameters, CDM scaffolds are capable of maintaining complex structures that support MSC chondrogenesis.

1. Introduction

In the field of cartilage tissue engineering, there is a growing interest in using the native cartilage extracellular matrix as a scaffold biomaterial due to its ability to retain active growth factors [1] as well as to provide cartilage-specific epitopes for cell-matrix interactions. Since initial reports that chondrocytes were capable of bonding devitalized articular cartilage slices [2], several studies have revealed that cartilage extracellular matrix extract can promote cartilage-specific differentiation of embryonic stem cells [3] and prevented dedifferentiation of chondrocytes [4,5]. The chondroinductive properties of cartilage-derived matrix (CDM) scaffolds have been demonstrated in a variety of cell types including: adipose-derived [6–11], synovium-derived [12], infrapatellar fat pad-derived [13,14], and bone marrow-derived stem cells (MSCs) [1,7,15–23], as well as chondrocytes [2,21,24–28]. Of particular note is the finding that, depending on the cell type, CDM can promote chondrogenic differentiation in the absence of exogenous growth factors [1,6,9,12,20,24,25], or exhibit a synergistic effect with growth factor supplementation [1,7,11–13,18,27,28]. CDM has also been shown to enhance the *in vivo* repair of cartilage defects

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[8,10,16,19,26]. Furthermore, studies comparing collagen-hyaluronic acid scaffolds to CDM constructs illustrated that CDM constructs retained newly synthesized glycosaminoglycans better than collagen-hyaluronic acid scaffolds [13].

In order to mitigate potential immunogenic responses towards foreign cellular material, most native tissue biomaterials are decellularized [12,15,19,22,29–33]. To facilitate the removal of cellular debris, CDM is often pulverized into a fine power [12,32], which can then be fabricated into a porous scaffold [14,19,21,26]. While this processing improves decellularization and enhances repopulation with seeded cells, these treatments dramatically reduce the mechanical properties of CDM scaffolds, making them susceptible to cell-mediated contraction [6,7,9,12–14,18,24,34]. This contraction unpredictably alters the shape of CDM constructs and limits the space available for cellular proliferation and matrix deposition.

Previous studies have implemented chemical crosslinking treatments [8,9,13–18,23,26] or reinforced CDM with synthetic polymers [34] to mitigate cell-mediated contraction. Physical crosslinking techniques such as dehydrothermal treatment [18,35,36] or ultraviolet irradiation [18,19,21,35,36] have also been applied to prevent cell-mediated contraction of CDM scaffolds. Furthermore, these physical crosslinking treatments have been shown to preserve epitopes that participate in cell-matrix interactions, and supported greater chondrogenic differentiation than chemically crosslinked scaffolds [18]. Therefore in the current study, we sought to minimize the manipulation of the native cartilage extracellular matrix by only using dehydrothermal treatment to minimally crosslink CDM constructs.

In this study, we developed a method for fabrication of anatomically relevant, hemispherical CDM constructs seeded with human MSCs. To enhance the mechanical properties of these constructs, we altered the CDM concentration [14] and pore architecture [23] of the CDM scaffolds. Previous studies demonstrated that increasing CDM concentration dramatically increased the compressive modulus of CDM constructs [14]. However, increasing CDM concentration also corresponded with a concomitant decrease in pore size, which restricted cells to the surface of CDM constructs at high CDM concentrations [14]. Aligning the pores of CDM scaffolds via unidirectional freezing has also been shown to enhance the compressive modulus of CDM constructs and facilitate cellular infiltration resulting in uniform cellular distribution [23]. While these studies elucidated the prominent roles of CDM concentration and pore architecture in governing the mechanical properties of CDM scaffolds, each group examined these variables separately and both studies required chemical crosslinking treatments in order to prevent cell-mediated contraction. The current study investigated the synergistic effect of CDM concentration and pore architecture on the mechanical properties of CDM scaffolds, their ability to prevent cell-mediated contraction, and their influence on cellular infiltration. Using this defined scaffold fabrication method, we created anatomically-shaped CDM hemispheres that were seeded with MSCs and underwent chondrogenic differentiation *in vitro*.

2. Methods

2.1. Preparation of scaffolds

Articular cartilage was harvested from the femoral condyles of freshly slaughtered, skeletally-mature (over 18 months of age), female pigs (n = 200). Cartilage was shaved off of the bone in large pieces, frozen overnight at −80 °C, and lyophilized (Freezone 2.5L, Labconco, Kansas City, MO) for 24 h. Lyophilized cartilage was pulverized into a fine powder using a 6770 freezer/mill (SPEX SamplePrep, Metuchen, NJ). Cartilage was precooled for 3 min prior to pulverization at 5 Hz for 10 cycles of 1 min run, 1 min cool. Cartilage powder was treated with 10 mM Tris-HCl (pH 7.5) containing 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 50 U/mL DNase I (Sigma, St. Louis, MO) at a ratio of 20 mL decellularization solution per 1 g cartilage powder for 24 h at 37 °C, adapted from Ref. [15]. In order to preserve the GAG content of the cartilage powder, decellularized CDM was immediately frozen and lyophilized after DNase treatment. The lyophilized CDM was pulverized again using the settings described above to form a fine powder, which was then sieved through a mesh with a 97 μm mesh size to ensure that all particles were at most 97 μm in one dimension. Decellularized CDM powder from each pig joint was combined to form a single superlot of powder (n = 200) that was used for all experiments. In order to produce CDM concentrations of 11%, 10%, 9%, 8%, and 7% weight/weight, cartilage powder was weighed into aliquots of 1.1, 1.0, 0.9, 0.8, or 0.7 g, respectively, then distilled water was added to each until a final weight of 10 g was reached. Cartilage powder was suspended in distilled water using a homogenizer (PRO260, PRO Scientific Inc., Oxford, CT). Cartilage was homogenized for five cycles of 2 min homogenization at 30,000 rpm and cooling 2 min on ice to prevent overheating. Homogenized cartilage was pipetted into one of the following two-part delrin-silicone molds: 1) discs 6 mm in diameter, 2 mm deep with a flat silicone lid 2) hemispheres having an outer radius of 4.76 mm with a silicone lid containing hemisphere protrusions to generate an inner radius of 3.175 mm (Fig. 1). In order to produce aligned pores, the two-part molds were placed directly into a −80 °C freezer and frozen overnight. Since the silicone lids were much thinner than the delrin molds, they froze first producing a temperature gradient during the freezing process, which aligned the ice crystals and thus the pores in the scaffold [23,37]. In order to produce uniform pores, the two-part molds were placed in a Styrofoam container filled with isopropyl alcohol and then placed in a −80 °C freezer, which cooled both halves of the mold at the same rate, thus removing the temperature gradient. After freezing overnight, the silicone lids were removed and scaffolds were lyophilized (Freezone 2.5L) for 24 h. After lyophilization, scaffolds were crosslinked and sterilized via dehydrothermal treatment by heating scaffolds in a dry climate at 120 °C for 24 h.

2.2. Porosity and pore size measurement

CDM discs were scanned using micro-computed tomography (micro-CT) (SkyScan 1176, Bruker, Billerica, MA) at 40 kV, 600 μA, 16.67 μm isotropic spatial resolution. Micro-CT datasets were reconstructed with NRecon software (Bruker) using a dynamic range of 0.0171, ring artifact correction of 11, and beam hardening correction of 20%. Reconstructed images were binarized using thresholds that were calibrated to the Archimedes-based volume fractions as described previously [38]. Porosity and pore size were calculated via image processing executed with CT-Analyzer software (Bruker).

2.3. Analysis of pore architecture

CDM scaffolds were sputter-coated (Desk IV, Denton Vacuum, Moorestown, NJ) with gold at 18 mA for 600 s, which resulted in a gold sputter deposition of 20 nm in thickness. Coated samples were scanned (FEI XL30 ESEM, Hillsboro, OR) at an accelerating voltage of 30 kV. For scaffolds seeded with cells prior to sputter coating, constructs were fixed in 2.5% glutaraldehyde (Electron Microscope Sciences, Hatfield, PA) for 15 min, dehydrated in a graded ethanol series to 100% ethanol, and critical point dried using hexamethyldisilazane (Electron Microscope Sciences).

2.4. Cell culture

Bone marrow was obtained in concordance with an approved