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Pore Architecture Effects on Chondrogenic Potential of Patient-Specific 3-Dimensionally Printed Porous Tissue Bioscaffolds for Auricular Tissue Engineering

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Abstract

Objective: This study aims to determine the effect of auricular scaffold microarchitecture on chondrogenic potential in an *in vivo* animal model.

Methods: DICOM computed tomography (CT) images of a human auricle were segmented to create an external anatomic envelope. Image-based design was used to generate 1) orthogonally interconnected spherical pores and 2) randomly interspersed pores, and each were repeated in three dimensions to fill the external auricular envelope. These auricular scaffolds were then 3D printed by laser sintering poly-L-caprolactone, seeded with primary porcine auricular chondrocytes in a hyaluronic acid/collagen hydrogel and cultured in a pro-chondrogenic medium. The auricular scaffolds were then implanted subcutaneously in rats and explanted after 4 weeks for analysis with Safranin O and Hematoxylin and Eosin staining.

Results: Auricular constructs with two micropore architectures were rapidly manufactured with high fidelity anatomic appearance. Subcutaneous implantation of the scaffolds resulted in excellent external appearance of both anterior and posterior auricular surfaces. Analysis on explantation showed that the defined, spherical micropore architecture yielded histologic evidence of more robust chondrogenic tissue formation as demonstrated by Safranin O and Hematoxylin and Eosin staining.

Conclusions: Image-based computer-aided design and 3D printing offers an exciting new avenue for the tissue-engineered auricle. In early pilot work, creation of spherical micropores within the scaffold architecture appears to impart greater chondrogenicity of the bioscaffold. This

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Disclosure

The authors have no conflicts of interest to disclose.

advantage could be related to differences in permeability allowing greater cell migration and nutrient flow, differences in surface area allowing different cell aggregation, or a combination of both factors. The ability to design an anatomically correct scaffold that maintains its structural integrity while also promoting auricular cartilage growth represents an important step towards clinical applicability of this new technology.

Keywords

auricular reconstruction; microtia; computer-aided design; computer-aided manufacturing; 3 dimensional printing; tissue engineering

1. Introduction

Auricular reconstruction is a demanding challenge for the reconstructive surgeon as it requires reproduction of composite soft tissue with high geometric complexity. Current clinical reconstruction options include the hand-carved framework fabrication using autogenous costal cartilage, the manufacturing of alloplastic porous polyethylene implants, or the use of an ear prosthesis. Significant risks and disadvantages exist for each available method and include extrusion of the implant, infection, and the need for multiple surgeries. [1] In addition, while autogenous cartilage grafts may represent the most promising reconstructive technique, the high degree of surgical skill required to produce a consistently successful outcome limits the broad adoptability of this method. Our group has previously demonstrated that 3-Dimensional (3D) printing – also known as additive manufacturing – integrated with image-based computer aided design enables rapid production of patient-specific anatomic soft tissue implants and tissue engineering bioscaffolds that can reproduce complex craniofacial structures with high fidelity [2–3,4,5]. Moreover, controlling the porous characteristics of the scaffolds can influence differentiation of pluripotent stem cells, suggesting that scaffold microarchitecture can be optimized for cartilage or bone. [6,7]

This study compared two different microporous scaffold architectures to demonstrate the importance of scaffold design in optimizing auricular cartilage growth. The pilot results suggest that a defined arrangement of spherical pores yielded more robust auricular tissue growth.

2. Materials and Methods

2.1 Scaffold design and manufacturing

Scaffolds were created using image-based hierarchical design methods previously described by our group [8–9,10,11,12,13,14]. Hierarchical image designs were created separately for the global ear structure. Designs were represented by a density distribution within a voxel format, similar to the way 3D images are represented by density distributions within a voxel dataset. Separate voxel design datasets were created for the anatomic structure, based on a patient's radiologic data. Different pore structures were created by generating either periodic or random geometries – such as spheres or cylinders – using density distributions in voxel data structures created by specially written MATLAB™ codes. This mapped the pore structure over a cube that encompassed the final size of the anatomic region. The voxel

structure was converted into a triangular surface .STL representation. The final bioscaffold design was created by mapping a porous architecture .STL file onto the appropriate location of the anatomic dataset (also represented as a .STL file after conversion in the commercial software MIMICS™, by Materialise). In this study, a single architecture, composed of either periodic spherical or random pores, was mapped into the global patient-specific anatomic design for the ear using Boolean intersection operations of the custom designed porous architecture .STL file and the ear .STL file using MIMICS to create the final scaffold design.

Our group's manufacturing[15–,16,17,18] approach utilizes laser sintered polycaprolactone (PCL), an FDA approved, bioresorbable polymer. Based on previous work, which established the powder size, bed temperature, and laser sintering power, an EOS P100 laser sintering system was used to fabricate patient-specific ear scaffolds.[16] In this report a 69% scaled male left ear was utilized. Random porosity was set at 61%. Spherical porosity was set at 65%. Pore size was 2.5mm.

2.2 In vitro cartilage growth

Institutional Animal Care and Use Committee protocol approval was obtained. Chondrocytes were isolated from freshly harvested porcine auricular cartilage. Care was taken to isolate cartilage while discarding overlying perichondrium. Minced cartilage fragments were digested with 0.2% type II collagenase (Worthington Biochemical, Lakeview, NJ) for 16 hours in a 37°C, 5% CO₂ incubator with agitation. The digest was filtered through a 70-micron mesh (Becton Dickinson, Franklin Lakes, NJ), and the cells were centrifuged to precipitate, counted, and plated. The proliferation medium consisted of Ham's F-12 (Gibco, BRL/Life Technologies, Grand Island, NY), with the addition of 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 5 mg/ml ascorbic acid, and an antibiotic/antimycotic solution containing 10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 ug/ml Fungizone.

Chondrocytes were seeded into the auricular PCL scaffolds using a type I collagen/hyaluronic acid composite gel, which we have shown previously supports chondrogenesis. [19] The gel solution consisted of type I collagen at a concentration of 6 mg/ml in acetic acid (Becton Dickinson, Franklin Lakes, NJ) and hyaluronic acid at a concentration of 3 mg/ml (LifeCore Biomedical, Chaska, MN). Cells were rinsed with Hanks Buffered Saline Solution (HBSS, Gibco, BRL/Life Technologies, Grand Island, NY), trypsinized (0.25% trypsin, Gibco), and aliquoted into 15 ml conical tubes and placed on ice. Prior to seeding, the PCL scaffolds were placed in custom-designed Sylgard® (Dow Corning, Midland, MI) molds to prevent leakage of the cell-collagen solution prior to gelation. After resuspending the cells in the collagen I gel solution, sodium bicarbonate was added, the cell suspension was carefully pipetted into the PCL scaffolds and the constructs were placed in an incubator (37°C, 5% CO₂) for 30 minutes for gelation to occur. Approximately 25×10^6 chondrocytes were utilized per scaffold. Seeded constructs were cultured in sterile, dynamic conditions with incubation at 37°C, 5% CO₂. The culture medium consisted of serum free F12 (Gibco), with the addition of 5 ng/ml TGF-2 (Pepro Tech, Rocky Hill, NJ), ITS+premix (Becon Dickinson), 110mm pyruvate (Gibco), 10µm dexamethasone (Sigma), and 5 µg/ml ascorbic acid.

2.3 In vivo scaffold implantation

After 4 weeks of *in vitro* culture, the chondrocyte-seeded scaffolds were implanted into 7–10 week-old NIH-Foxn1 strain 316, Charles River athymic rodents. General anesthetic was administered. A dorsal incision was performed with development of subcutaneous pocket. Layered skin closure was performed with 4–0 monocryl subcuticular closure.

After 9.5 weeks, ear constructs were removed and histologically analyzed. For histology, one random and one spherical ear scaffold were divided into quarters. The specimens were fixed with 10% phosphate buffered formalin for 24 hours, and then embedded in paraffin and sectioned using standard histochemical techniques. Serial slide sections were stained with hematoxylin and eosin (H&E) or Safranin O.

3. Results

Auricular constructs with two micropore architectures, random and spherical, were rapidly manufactured with high fidelity anatomic appearance (Figure 1). Subcutaneous implantation of the bioscaffolds resulted in excellent external appearance of both anterior and posterior auricular surfaces. Scaffold landmarks including helix, antihelix, conchal bowl, tragus, antitragus, and intertragal incisor were readily evident after subcutaneous implantation. The anatomic subunits and overall dimensions of the ear were preserved for the duration of the *in vivo* analysis and until necropsy. Projection was approximately 25–30 ° off horizontal plane of the animal dorsum (Figure 2).

Histologic analysis displayed more robust Safranin O staining for the spherical scaffolds in comparison to random pore scaffolds, indicating a greater amount of cartilage tissue. Engineered cartilage tissue was seen in both the peripheral and central aspects of the auricular scaffolds. Tissue growth was observed adjacent to the polycaprolactone structures, although there was no aberrant cartilage growth as it did not extend beyond the confines of the scaffold (Figure 3). H&E staining also showed more robust cartilage tissue growth in the spherical scaffolds.

4. Discussion

Auricular reconstruction poses a unique challenge for the reconstructive surgeon. Research has demonstrated that there is significant psychosocial morbidity associated with auricular deformity and that ear reconstruction can provide substantial improvements to quality of life in both adult and pediatric patients. In one study, over 90% of children who underwent auricular reconstruction reported improved self-confidence and social life. [20] Despite this known clinical importance of auricular reconstruction, currently available techniques each have their own limitations. In reconstruction with rib cartilage, disadvantages are numerous. These include the technical difficulty of the surgery, the donor site morbidity, and the need for multiple surgical procedures alongside the associated risks of general anesthetic exposures and pneumothorax. Moreover, the requirement of adequate costal cartilage volume can significantly delay the age of reconstruction. A study demonstrated a superior aesthetic outcome with use of porous polyethylene as compared to

an equal number of rib cartilage reconstructions, suggesting that successful reconstruction with rib cartilage is difficult to achieve.[1]

Disadvantages associated with porous polyethylene implants include significant cost, higher rates of extrusion, fracture, and infection.[1,21] Complication rates are likely underreported and ill-defined.

While prosthetic devices may have excellent aesthetics, they wear, requiring frequent replacement. They also require significant care and have the potential for accidental dislodgement which decreases their attractiveness. Ultimately, they are not a durable solution.[22]

Tissue engineering has attempted to provide an alternative to the current clinical options. One of the first notable attempts utilized a negative Crayola clay ear mold to pack bioresorbable polymer to successfully produce an auricular shaped cartilage.[23] A review of this work summarizes a number of studies that have experimented with a variety of tissue bioscaffolds, cell sources, growth factors and cell culture methods.[24] The advances made in auricular cartilage tissue engineering have also revealed potential clinical concerns, such as framework contraction and limitations in attaining the large cell numbers required to populate tissue scaffolds. Methods for feasible clinical implementation have not yet been described.

A decade has passed since the seminal work in cartilage tissue engineering was first performed, and this study seeks to continue to improve the technology by describing an approach that carries a number of potential benefits. Similar methods were recently described to treat life threatening tracheobronchomalacia in an animal model and human case. [25,26] Selective laser sintering enables the use of photographically or radiographically obtained patient-specific imaging data to create custom 3D-printed bioscaffolds, which represents a marked improvement over generic, prefabricated porous polyethylene implants. Furthermore, by imparting meticulous pore architectures to these specifically designed scaffolds, cellular infiltration, biomechanics, and tissue growth can be optimized to improve construct viability while maintaining sufficient mechanical stiffness to resist contraction.

One aim of this study was to further elucidate the potential benefit of utilizing a defined, as opposed to random, pore design. Previous work by our group has demonstrated the ability to direct pluripotent bone marrow derived stem cells toward either an osteocyte or chondrocyte differentiation by adjusting pore design.[7] In this study, 3D printed ear scaffolds were rapidly and consistently manufactured from bioresorbable polycaprolactone. Although aesthetic appearance was not a primary goal of the study, the ear scaffolds revealed and maintained excellent anatomical detail when subcutaneously implanted. We suspect appearance may be further improved with use of negative pressure suction.

Cartilage growth was observed in both scaffold designs, but appeared more robust in the spherical pore design. This supports previous studies suggesting that a spherical pore design encourages chondrocytes to adopt their preferred conformation and proceed with matrix deposition. [6] The interconnected pore design may also provide higher permeability conducive to cell nutrition.[18] Furthermore, cartilage growth appeared equally in the

peripheral and central regions of the scaffolds, but did not appear to grow beyond the boundaries of the scaffold. This suggests that the cells seeded on the scaffold prior to implantation remained confined within the scaffold and promoted tissue growth within the construct, as intended.

Though these findings provide evidence that tissue engineering may be an attractive alternative to existing auricular reconstruction methods, additional work is necessary. Our aim in reporting this early pilot work is to potentially help guide ongoing efforts toward developments in auricular tissue engineering. Our group is engaged in longer term studies necessary to assess the construct shape resilience and to characterize construct resorption. These studies are currently underway and aim to create bioscaffolds with precisely defined architecture that both resist contraction and allow for gradual replacement with native cartilage matrix.

5. Conclusions

Image-based, patient-specific computer-aided design and 3D printing offers an exciting new avenue for auricular cartilage tissue engineering. Creation of spherical micropores within the scaffold architecture appears to impart greater chondrogenicity to the scaffold in this early pilot work. The ability to design an anatomically correct bioscaffold that maintains its structural integrity and promotes auricular cartilage growth represents an important step towards the clinical applicability of this new technology.

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Figure 1: Random and pore ear scaffolds before, during, and after seeding.
Random and spherical pore ear scaffolds (left). Scaffold in Sylgard® mold (mid). Scaffold seeded with chondrocytes in a collagen-hyaluronic acid hydrogel (right).

Figure 2: Ear scaffolds after subcutaneous implantation.

Posterior surface up on right dorsum, anterior surface up on left dorsum (left). Anterior face surface details (right upper) and anterior oblique view highlighting excellent projection (right lower).

Figure 3: Transverse sections of auricular tissue scaffolds with Safranin O staining represents cartilage growth.
Random pore architecture (upper four panels); spherical pore structure (lower four panels).