

Title: Co-Culture of Adipose-Derived Stem Cells and Chondrocytes on Three-Dimensionally Printed Bioscaffolds for Craniofacial Cartilage Engineering

Short Running Title: Co-Culture on 3D-Printed Bioscaffolds

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Abstract

Objectives/Hypothesis. Reconstruction of craniofacial cartilagenous defects are among the most challenging surgical procedures in facial plastic surgery. Bioengineered craniofacial cartilage holds immense potential to surpass current reconstructive options but limitations to clinical translation exist. We endeavored to determine the viability of utilizing adipose-derived stem cell-chondrocyte co-culture and three-dimensional (3D) printing to produce 3D bioscaffolds for cartilage tissue engineering. We describe a feasibility study revealing a novel approach for cartilage tissue engineering with *in vitro* and *in vivo* animal data.

Methods. Porcine adipose-derived stem cells and chondrocytes were isolated and co-seeded at 1:1, 2:1, 5:1, 10:1, and 0:1 experimental ratios in a hyaluronic acid/collagen hydrogel in the pores of 3D-printed polycaprolactone scaffolds to form 3D bioscaffolds for cartilage tissue engineering. Bioscaffolds were cultured *in vitro* without growth factors for 4 weeks then implanted into the subcutaneous tissue of athymic rats for an additional 4 weeks before sacrifice. Bioscaffolds were subjected to histologic, immunohistochemical, and biochemical analysis.

Results. Successful production of cartilage was achieved using a co-culture model of adipose-derived stem cells and chondrocytes, without the use of exogenous growth factors. Histology demonstrated cartilage growth for all experimental ratios at the post-*in vivo* time point confirmed with type II collagen immunohistochemistry. There was no difference in sulfated-glycosaminoglycan production between experimental groups.

Conclusion. Tissue engineered cartilage was successfully produced on 3D-printed bioresorbable scaffolds using an adipose-derived stem cell and chondrocyte co-culture technique. This potentiates co-culture as a solution for several key barriers to a clinically-translatable cartilage tissue engineering process.

Keywords: auricular reconstruction; microtia; anotia; nasal reconstruction; computer-aided design; computer-aided manufacturing; CAD/CAM; three-dimensional printing; tissue engineering; craniofacial reconstruction

Level of Evidence: NA

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INTRODUCTION

Reconstruction of craniofacial cartilagenous defects, performed for an unsalvageable auricular or nasal framework in the setting of trauma, oncologic resection, or congenital malformation, are among the most technically challenging surgical procedures in facial plastic surgery¹. A select number of surgeons have mastered the art of using autologous rib cartilage, the current gold standard, to carve a neo-framework resulting in aesthetically and functionally acceptable results²⁻³. Alternately, some surgeons have elected to use synthetic constructs fashioned in the shape of an auricle or nose⁴⁻⁵.

The field of cartilage tissue engineering (CTE) holds immense potential for creation of bioengineered craniofacial cartilage that both surpasses current reconstructive options and decreases patient morbidity. Several promising studies have demonstrated the use of chondrocytes or chondrogenically pulsed mesenchymal stem cells (MSCs) to produce neocartilage that is histologically and mechanically comparable to native auricular cartilage⁶⁻⁹.

These techniques can be combined with patient-specific computer-aided design (CAD) and three-dimensional (3D) printing to produce high-fidelity auricular and nasal tissue engineering scaffolds¹⁰. However, with the many positive prospects in CTE, significant challenges remain, most notably the need for a large (10^7) number of cells.

Co-culture, where chondrocytes and MSCs are simultaneously seeded onto tissue engineering scaffolds, is a new technique that holds promise for circumventing some of the current limitations of utilizing chondrocytes or MSCs alone. Here we report the use of a co-culture model using adipose-derived stem cells (ASCs) and chondrocytes for CTE. We hypothesize that

co-culture technique can be adapted for craniofacial cartilage applications using hydrogels combined with 3D-printed bioresorbable scaffolds and that a variety of ratios of ASCs-to-chondrocytes may be utilized. This approach affords the potential for patient-specific CTE using computer-aided design (CAD) while mitigating the limitations of cell availability and need for prolonged in vitro cell culture or exogenous growth factor exposure of traditional CTE approaches. These represent key barriers to the eventual goal of creating a clinically translatable patient-specific craniofacial CTE methodology. Our objective is to assess the feasibility of this approach and the effect of ASC-to-chondrocyte ratio on cartilage production using histologic, immunohistochemical, and biochemical analysis.

MATERIALS AND METHODS

Protocol approval was obtained by the University of Michigan Institutional Animal Care & Use Committee and the University of Illinois Institutional Animal Care & Use Committee (University of Michigan #3857, University of Illinois #10114).

Scaffold Design and Manufacturing via 3D Printing

Scaffolds were created using previously described image-based hierarchical design methods developed by Hollister and colleagues¹¹⁻¹⁷. This process can be used to create patient-specific tissue engineering scaffolds of any geometry¹⁰. A standard 10 mm x 5 mm cylindrical disc scaffold macroarchitecture with a 2.7 mm spherical pore internal microarchitecture was chosen for this study to produce consistency of constructs for tissue analysis (Figure 1A). This yielded an overall scaffold porosity of 68.3% with an available volume per scaffold of 268 μ L. A midline groove was incorporated into the scaffold to facilitate bifurcation during analysis. The final

scaffold design was then 3D printed using an EOS P100 laser sintering system (EOS North America, Novi, Michigan) adapted to laser sinter L-polycaprolactone (PCL) powder (PCL Source: Polysciences, Warrington, Pennsylvania; PCL Preparation: Jet Pulverizer, Moorsetown, New Jersey)¹⁸. The laser sintering process can accurately reproduce feature sizes on the order of 700 μm and produce over 500 representative scaffolds with a single print cycle (Figure 1B). Scaffolds were cleaned of residual excess powder via sonication in 70% sterile ethanol then sterilized in a 24 hour 70% sterile ethanol soak prior to use.

Cell Harvest and Culture

Porcine ASCs derived from subcutaneous back fat and chondrocytes derived from auricular and tracheal cartilage were harvested from adolescent Yorkshire pigs using the methods previously developed by Wheeler and colleagues¹⁹⁻²⁰. Porcine cells were utilized for experiments due to the investigators' experience with these cells and availability of the necessary cell types. Primary (P0) ASC and chondrocyte cells were spun down and frozen prior to cell seeding experiments.

At the time of preparation for scaffold seeding, cells were thawed and expanded in growth media consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS) (Gibco), 1% pen/strep, and 0.2% Fungizone in a 37°C, 5% CO₂ incubator. ASCs were expanded to passage 2 (P2) and chondrocytes expanded to passage 1 (P1) to provide sufficient cells for seeding. Cells were passaged at 90% confluence.

Creation of Experimental Ratios and Scaffold Seeding

Adipose-derived stem cells and chondrocytes were rinsed with Hank's buffered saline solution (HBSS) (Gibco), trypsinized (0.25% trypsin) (Gibco), and aliquoted into experimental ratios of

1:1, 2:1, 5:1, 10:1, and 0:1 ASC-to-chondrocytes. Cell counting was performed prior to cell mixing using an automated cell counter (Moxi Z, Orflo Technologies). Given that the cells were harvested from several animals, each cell type was pooled prior to creation of experimental ratios. Each experimental group was then re-suspended in a type I collagen:hyaluronic acid hydrogel solution and seeded into a pre-wetted cylindrical PCL scaffold²¹. The hydrogel consisted of type I collagen at a concentration of 6 mg/mL in acetic acid (Discovery Labware) and hyaluronic acid at a concentration of 3 mg/mL (LifeCore Biomedical). Prior to seeding, the PCL scaffolds were placed in custom-fabricated silicone (Sylgard, Dow Corning) molds to prevent extravasation of the seeding solution prior to gelation. A 0.05N NaOH in NaCO₃ solution was used to induce gelation and scaffolds were subsequently transferred to 24-well low attachment plates (Fischer Scientific) for culture. The cell seeding density was 2×10^6 cells/cm³ and total of 12 scaffolds per experimental group were seeded.

In Vitro Incubation

Seeded constructs were placed individually within a 24-well low attachment plate with 1 scaffold per well and placed in a sterile CO₂ incubator. Scaffolds were incubated at 37°C with 5% CO₂ on an orbital shaker within the incubator for 4 weeks. Culture media was changed every 2-3 days by aspirating old media from each culture well and replacing with 1.25 mL of fresh media warmed to 37°C within a sterile culture hood. Culture media consisted of low-glucose DMEM with 10% FBS, 1% pen/strep, and 0.2% Fungizone. After 4 weeks, six scaffolds from each experimental group were extracted for post-*in vitro* biochemical analysis, while the remaining six were reserved for *in vivo* implantation.

In Vivo Implantation

Seven athymic rats underwent implantation with tissue engineering scaffolds under general anesthesia with isoflurane delivered by mask. All scaffolds were rinsed with HBSS prior to implantation. All animals were male with each weighing between 250 and 305 g. Each animal was shaved, prepped with iodine solution after induction of anesthesia and a vertical incision was sharply made on the dorsum of the animal. A total of 4 scaffolds per animal were implanted in a subcutaneous pocket into randomized quadrants on the back of the animal. The incision was then closed with surgical staples, which were removed on post-operative day seven. After 4 weeks, the animals were euthanized and the scaffolds were harvested for post-*in vivo* analysis.

Biochemical Analysis

Post-*in vitro* and post-*in vivo* specimens were split along the midline groove to double the number of constructs for analysis. One-half of each construct was weighed wet, lyophilized, reweighed dry, and digested in 1 mg/mL Papain stock solution (Fischer Scientific) at 65°C for 16 hours. PicoGreen assay (Invitrogen, Molecular Probes) was used to quantify the DNA content of the constructs with Lambda phage DNA (0-1 mg/mL) as a standard. The sulfated-glycosaminoglycan (s-GAG) content was measured using the Blyscan Glycosaminoglycan Assay (Accurate Chemical & Scientific Corp).

Histology and Immunohistochemistry

Remaining post-*in vivo* constructs were fixed in 4% formalin for 24 hours, embedded in paraffin (TissuePrep, Fischer Scientific), and processed using standard histologic procedures with a slice thickness of 10 μ m. Stains included hematoxylin and eosin, Safrinin-O, and toluidine blue.

Type II collagen immunohistochemical staining was performed using 5 $\mu\text{g}/\text{mL}$ primary mouse anti-type II collagen monoclonal antibodies (Developmental Studies Hybridoma Bank, University of Iowa).

Statistical Analysis

Data for biochemical analysis (DNA and s-GAG expression) were collected from six samples per co-culture experimental group after 4 weeks of *in vitro* cell culture and 4 weeks of *in vivo* growth. Data was expressed as mean \pm standard error of the mean (SEM). Results were analyzed using Student's t-test using SPSS 17.0 (SPSS Inc., Chicago, IL) and statistical significance was set to 5% ($\alpha=0.05$) in all analyses.

RESULTS

In vitro co-culture of porcine ASCs and chondrocytes in 3D-printed PCL cylindrical discs with an internal spherical porous architecture resulted in growth and maintenance of cartilage-like tissue after 8 weeks (4 weeks *in vitro* + 4 weeks *in vivo*). Surgical implantation was straightforward and the scaffolds were well tolerated by the animals with no minor or major complications. There was good maintenance of structural support by the PCL scaffolds after 4 weeks growth in a subcutaneous pocket, as shown in Figure 1C. Histologically normal appearing cartilage growth was noted in all experimental co-culture groups after 1 month of *in vivo* culture, which was confirmed with type II collagen immunohistochemistry (Figure 2). Notably, the 5:1 ASC-to-chondrocyte ratio demonstrated well delineated hyaline cartilage architecture in histology, with dense collagen deposition and lacunae surrounding the chondrocytes and differentiated ASCs within the microspheres of the scaffold (Figure 3-4)

Biochemical analysis results are summarized in Figure 5. There was no statistically significant difference in s-GAG content normalized to scaffold wet weight ($\mu\text{g}/\text{mg}$) or DNA content ($\mu\text{g}/\text{mg}$) between the co-culture experimental groups at the post-*in vitro* or post-*in vivo* time points. There was a statistically significant higher s-GAG content normalized to scaffold wet weight ($\mu\text{g}/\text{mg}$) and DNA content ($\mu\text{g}/\text{mg}$) in all co-culture groups compared to the chondrocyte-alone control group ($p < 0.05$ for all analyses) at the post-*in vitro* timepoint, however this difference disappeared at the post-*in vivo* timepoint.

DISCUSSION

Reconstruction of the auricular and external nasal frameworks, whether performed in the setting of trauma, oncologic resection, or congenital malformation, are some of the most demanding procedures in facial reconstructive surgery. Few surgeons have mastered the art of using autogenous rib cartilage, the current gold standard, to carve a neo-auricular framework resulting in aesthetically pleasing results^{2,3}. Moreover, this approach typically requires multiple revision procedures and carries risks of donor site morbidity, pneumothorax, undesired scarring, and graft failure or infection. Alternately, some surgeons elect to use synthetic porous high-density polyethylene (MedPor, Styker Corp., Kalamazoo, Michigan) for reconstruction⁴. This rigid, synthetic material can be fashioned into a neo-auricular framework for sub-cutaneous implantation. However, rates of framework extrusion through and necrosis of the overlying skin while poorly reported, occur at a rate of at least 3-5%⁴.

Tissue engineering holds several ubiquitous advantages, including the ability to create a patient-specific living construct using the patient's own cells. Several promising studies have demonstrated the use of chondrocytes or MSCs cultured in pro-chondrogenic growth factors (GFs) as being able to develop neocartilage that is histologically and mechanically similar to native ear cartilage⁶⁻⁹. In these models, chondrocytes or MSCs are harvested, passaged and expanded, then seeded onto a scaffold and cultured *in vitro* prior to sub-cutaneous implantation. A variety of scaffold materials, including polyglycolic acid, polycaprolactone, chitosan, and silk, have been used successfully with varying properties that are favorable for different conditions⁶⁻⁹. However, with the many positive prospects of producing a tissue engineered auricle, new challenges have also surfaced.

The primary limitation of utilizing solely chondrocytes for CTE is the large number of cells (up to 5×10^7) needed to seed human-sized craniofacial frameworks^{9,22-26}. The number of chondrocytes available from autologous cartilage is limited and passaging chondrocytes induces dedifferentiation with loss of type II collagen and sulfated glycosaminoglycan (s-GAG) production²⁷. Mesenchymal stem cells, of which ample cell quantities are available, have been posited as a solution to seeding requirements. Prior experiments have shown a variety of MSC types, including ASCs and bone-marrow stromal cells, to be capable of chondrogenic differentiation²⁸⁻²⁹. However, chondrogenic commitment of MSCs requires exogenous delivery of GFs for weeks and cells can demonstrate a propensity for ossification³⁰⁻³¹. Additionally, neovascularization of 3D tissue engineered cartilagenous constructs has proven to be a challenge to the long-term stability of these constructs, particularly with fragility of ASCs in hypoxic tissue environments³².

Co-culturing of chondrocytes and MSCs is a new technique that holds promise for circumventing some limitations of utilizing chondrocytes or MSCs alone. In a co-culture model, chondrocytes and MSCs are simultaneously seeded onto a tissue engineering scaffold. Chondrocytes have been found to induce chondrogenic differentiation of the MSCs via production of exogenous GFs such as cytokine-like protein 1 (Cyt11), bone morphogenic protein-2 (BMP-2), parathyroid hormone-related peptide (PTHrP), and transforming growth factor-beta (TGF- β) as well as paracrine, juxtacrine, and gap-junction signaling pathways³³⁻³⁶. In this way, chondrocytes maintain the chondrogenic niche required for commitment of MSCs to the chondrogenic phenotype, circumventing the need for exogenous GF delivery. Additionally, chondrocytes provide matrix for MSC migration and prevent ossification of MSC-derived chondrocytes³⁷.

Our group has previously developed a process utilizing image-based design and 3D printing to produce high-fidelity patient-specific tissue engineering scaffolds using PCL, a bioresorbable polymer¹⁰. Utilization of a bioresorbable material allows for eventual replacement of the scaffold with chondrocyte extracellular matrix, thus best emulating natural craniofacial cartilage. We have previously seeded these scaffolds with primary chondrocytes to produce tissue engineered auricular and nasal constructions¹⁰. One advantage of a scaffold-specific CAD approach utilizing 3D-printed biomaterials is the ability to generate an intra-scaffold environment more conducive to cell survival and neovascularization. Our group has previously performed studies demonstrating that a spherical pore microarchitecture results in improved cell survival and cartilage deposition for CTE³⁸. This process affords the ability to rapidly produce high-fidelity anatomic scaffolds while also allowing meticulous control of the pore microarchitecture.

However, our prior investigations have been limited by many of the previously described constraints, including the limited number of chondrocytes available for harvest, the need to passage chondrocytes in cell culture, and the need for prolonged exogenous GF exposure.

Although other studies have utilized ASC/chondrocyte co-culture for CTE³⁹, this report is the first to our knowledge describing successful use of ASC co-culture on 3D-printed tissue engineering scaffolds for successful CTE. The use of ASCs with a co-culture technique is particularly advantageous for a clinically-translatable approach, given the low morbidity to harvest these cells vis-à-vis to bone-marrow derived stromal cells. The described process can readily be adapted for tissue engineering constructs of any shape, including patient-specific auricular and nasal constructs using DICOM data (Figure 6)¹⁰.

Our results demonstrate that all the experimental ratios of ASC-to-chondrocytes in our study resulted in type II collagen and cartilage production on short term *in vivo* analysis. Notably, this was achieved without the use of exogenous GFs during scaffold incubation. Using a cell count goal of 5×10^7 as the number of cells needed for a typical human-sized auricle, ratios of 10:1 and 5:1 ASC:PC yield cell number requirements which are clinically achievable from a cell harvest without the need for prolonged passaging of cells in the laboratory setting⁴⁰. These represent important barriers to a clinically-translatable process for craniofacial CTE which appear to be overcome with a co-culture technique.

Interestingly, the co-culture scaffold groups all appeared to perform similarly, despite different ratios of ASC-to-chondrocytes. Additionally, the co-culture groups appeared to outperform the chondrocyte-alone scaffolds in amount of histologic cartilage deposition, despite identical cell seeding densities. This may represent an inherent superior viability of co-cultured cells in this methodology or synergistic interaction of co-cultured cells to promote chondrogenesis.

However, this could also be an artifact of decreased viability of chondrocytes in cell culture. Given that no analysis of cell viability or gene expression was performed in this study, these ideas remain speculative.

Our feasibility study is limited by a short *in vivo* incubation period and small number of constructs. As such, statistical differences of the biochemical characteristics of the experimental groups may have not been captured, as well as differences in the trajectory of tissue deposition with more prolonged *in vivo* growth. Inferences regarding human translatability of a tissue engineering model utilizing porcine cells should be tempered until similar results can be demonstrated using human-derived PCs and ASCs. Our PCL constructs are anticipated to maintain construct fidelity for 2 to 3 years prior to resorption. More prolonged *in vivo* study will be necessary to determine whether the tissue engineered construct contracts or resorbs after scaffold dissolution. Additional study of the microscopic orientation and mechanical characteristics of ASC:PC co-cultured constructs will help delineate whether co-cultured cartilage creates primarily elastic cartilage or fibrocartilage, although this is of less importance in a structure such as an auricle. Further cell viability and gene expression analysis studies would also help characterize the chondrogenic commitment of ASCs and ability of ASCs and PCs to tolerate co-culture on 3D-printed bioscaffolds. We chose an incubation period of 4 weeks for

our scaffolds after cell seeding in concordance with previously described CTE techniques. However, given the lack of a need for exogenous GF delivery with a co-culture approach, we are currently investigating a “seed and go” approach where scaffolds are implanted immediately after cell seeding. Given that our MSCs were not immunofluorescently labeled, direct demonstration of chondrogenic differentiation of the MSCs was not possible, nor was it possible to assess migration of host MSCs into the constructs. However, we feel that histologic markers are a sufficient surrogate in the scope of this experiment given prior work which has demonstrated the successful chondrogenic commitment of MSCs using this technique.

CONCLUSION

We describe the successful use of an ASC-chondrocyte co-culture technique and CAD-designed 3D-printed tissue engineering scaffolds for CTE in an animal model. This co-culture model produced formal cartilage production on short term *in vivo* follow-up in all experimental groups, including 5:1 and 10:1 ASC:chondrocyte ratios. The clinical availability of ASCs and lack of a need for prolonged exogenous GF exposure suggest this approach mitigates many of the limitations of traditional CTE approaches. These represent key barriers to the eventual goal of creating a clinically translatable patient-specific craniofacial CTE methodology that may be overcome using co-culture and 3D printing.

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FIGURE LEGENDS

Figure 1. Computer-Aided Design and Three-Dimensional Printing Process for Production of Porous Bioresorbable Tissue Engineering Scaffolds. (A) Rendering of stereolithography (.STL) file for cylindrical tissue engineering scaffold with 2.7 mm spherical pore internal microarchitecture. (B) Final tissue engineering scaffold manufacturing via selective laser sintering three-dimensional printing technique. Scaffold features down to 70 μm can be successfully reproduced with this approach. (C) Final tissue engineering scaffolds after cell seeding in a hyaluronic acid/collagen hydrogel implanted into four randomized quadrants in a subcutaneous pocket on the dorsum of an athymic rat.

Figure 2. 40X histologic and immunohistochemical results of co-culture experimental groups at differing ratios of ASCs-to-chondrocytes after 4 weeks of *in vitro* followed by 4 weeks of *in vivo* culture. Representative slices are shown of each experimental group through the inner sections of the tissue engineering scaffolds at 40X magnification. ASC = Adipose-derived stem cells. Black scale bar in all panels = 300 μm .

Figure 3. 100X Safranin O stain of 5:1 ASC:chondrocyte co-culture scaffold after 4 weeks *in vivo* growth representative of a longitudinal inner section of a construct. White asterisk denotes well defined lacuna around chondrocytes within cartilage matrix. ASC = Adipose-derived stem cells. Black scale bar = 100 μm .

Figure 4. 40X type II collagen immunohistochemical stain of 5:1 ASC:chondrocyte co-culture scaffold after 4 weeks in vivo growth representative of a transverse outer section of a construct. ASC = Adipose-derived stem cells. Black scale bar = 1 mm.

Figure 5. Biochemical characterization for all experimental groups. All values expressed as mean values. All ratios expressed as adipose-derived stem cells-to-chondrocytes. Error bars represent standard error of the mean. s-GAG = sulfated glycosaminoglycan. DNA = dioxynuclease.

Figure 6. Computer-Aided Design and Three-Dimensional Printing Methodology for Patient-Specific Cartilage Tissue Engineering. DICOM data of the anatomic structure of interest is acquired and used to generate a three-dimensional model of the structure. The model is then converted into a porous structure using negative Boolean operations and manufactured from polycaprolactone using a selective laser sintering three-dimensional printer. The bioresorbable scaffold is then seeded with cells suspended in a hyaluronic acid/collagen hydrogel prior to implantation. (Adapted from *Otolaryngology-Head & Neck Surgery*, Volume 152, Issue 1, DA Zopf, AG Mitsak, CL Flanagan, et al. Computer-aided-designed, 3-dimensionally printed porous tissue bioscaffolds for craniofacial soft tissue reconstruction, 57-62, Copyright (2015), with permission from SAGE Publishing).

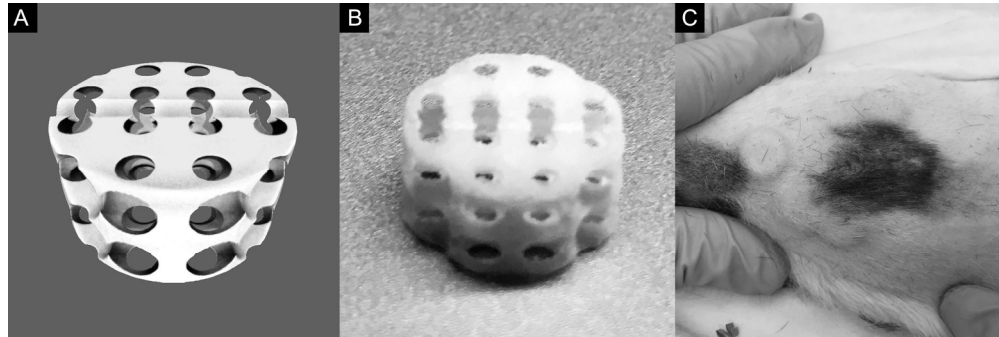


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756x251mm (72 x 72 DPI)

Author Ma

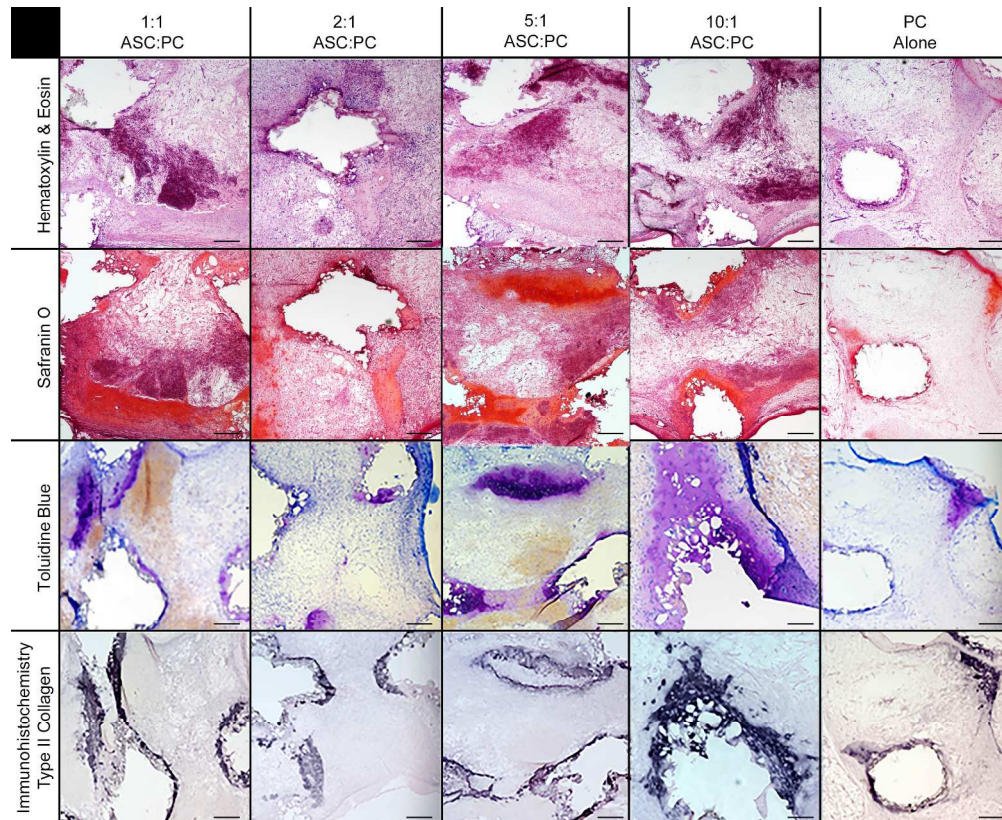


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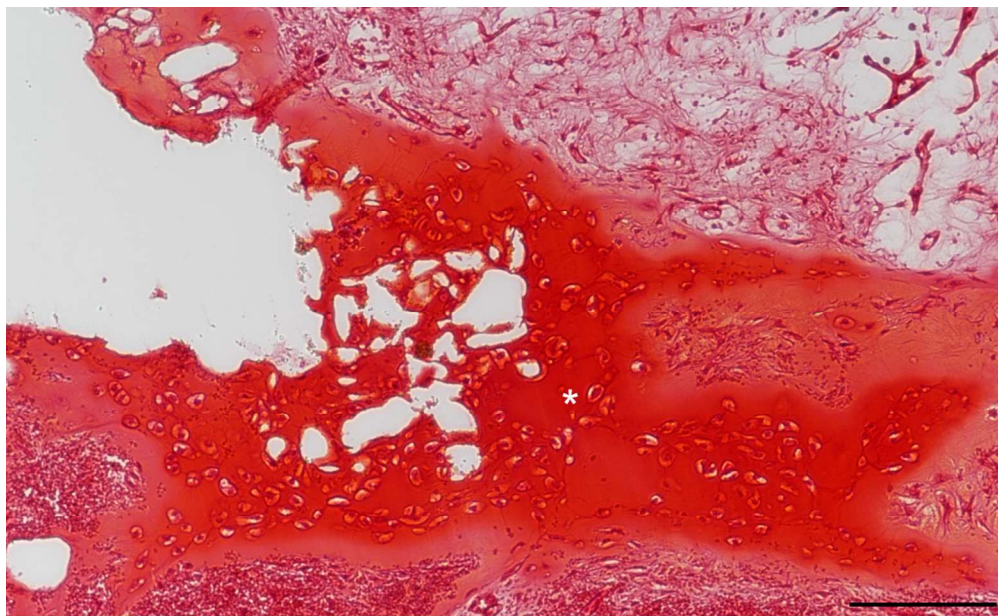


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432x263mm (72 x 72 DPI)

Author N

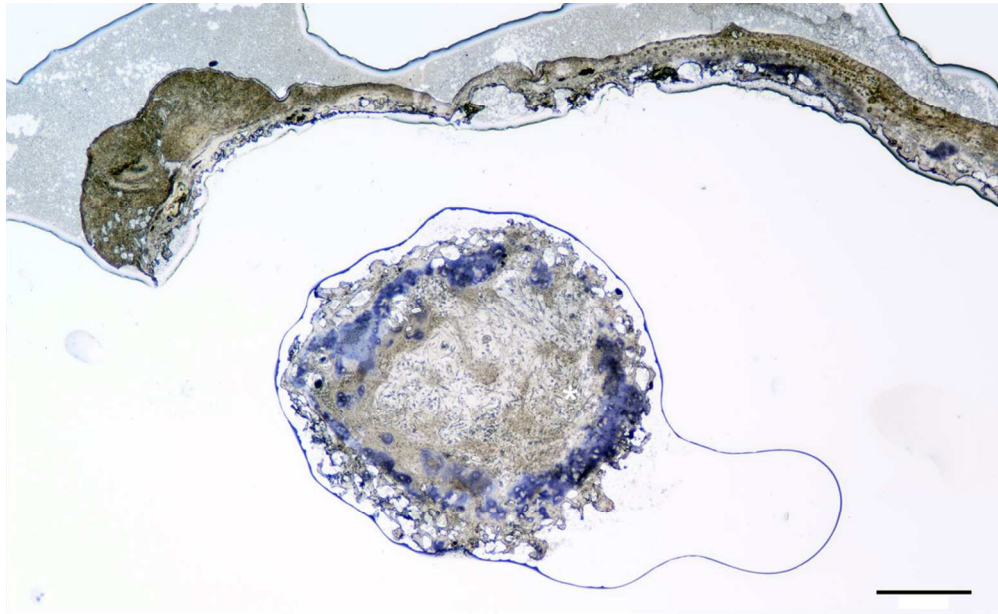


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432x263mm (72 x 72 DPI)

Author N

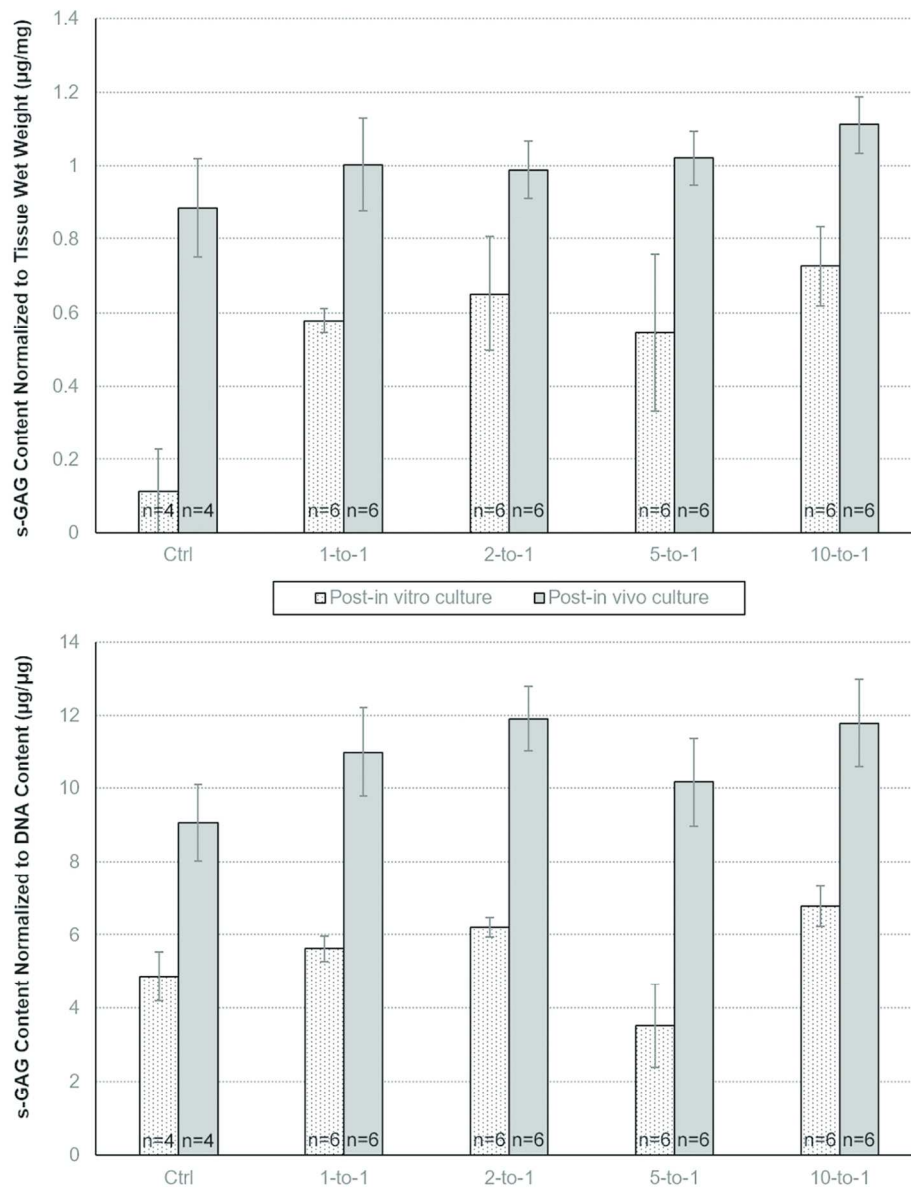


Figure 5. Biochemical characterization for all experimental groups. All values expressed as mean values. All ratios expressed as adipose-derived stem cells-to-chondrocytes. Error bars represent standard error of the mean. s-GAG = sulfated glycosaminoglycan. DNA = deoxyribonucleic acid.

127x160mm (300 x 300 DPI)

AJ



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165x19mm (300 x 300 DPI)

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