Title
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Journal
Berkeley Scientific Journal, 19(1)

ISSN
1097-0967

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Publication Date
2014

DOI
10.5070/BS3191025164

Peer reviewed|Undergraduate

Decelluarization and Reseeding of Porcine Colon with Human CaCo-2 Cells for Whole-Scale Organ Engineering Applications

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Keywords: tissue engineering, organ engineering, colon, enterocytes, regenerative medicine

Abstract

A new approach to whole-scale organ engineering is the seeding of stem cells upon a decellularized scaffold of a desired organ. Current efforts seek to use this method for whole-scale organ engineering. Here, we report a novel decellularization-recellularization approach to engineering a human colonic graft with human enterocytes and xenogeneic scaffold. Porcine colon tissue was decellularized with protein-solubilizing agents and recellularized by incubation with Caco-2. Immunohistochemistry, DNA staining, and SEM were used to study the effects upon the scaffold. Exposure to 2%SDS-0.5%Triton solution for four hours resulted in the best decellularization and preservation of microstructure, while tissues treated with 2%SDS-0.5%Triton and 0.05%Trypsin/EDTA supported the best recellularization. Our method provides a potential strategy for streamlined regeneration of a viable colonic lumen from xenogeneic and allogeneic sources while maintaining colon microstructure and potential for recellularization.

Introduction

The human colon is necessary for water, salt, and fat-soluble vitamin absorption. It also supports a rich microbial community that metabolizes undigested substances passing through the tract. The colon must also store and pass fecal matter out of the body. Colon injury, which can take a significant toll upon the human body, arises from a number of etiologies, including colorectal cancer (the third leading type of cancer, of which 102,480 new cases arise in the US per year (1)), Crohn's disease, and ulcerative colitis (both of which currently affect as many as 700,000 Americans (2)). Current treatment options include immunosuppressants (3) and ostomy pouching systems (4) . Previous efforts to engineer a replacement colon have been limited to digesting and centrifuging colons from rats before cell implantation onto scaffold tubes (5).

Current efforts in colon engineering have been limited to proof-of-concept demonstrations of decellularization of xenogeneic colon sources (6, 7, 8, 9). Here, we demonstrate the decellularization of xenogeneic colonic tissue and its recellularization with a human enterocyte line in order to create a graft of colonic lumen. The lumen is the innermost and main absorptive unit of the colon, and its fabrication serves as a proof-of-principle that our method can fabricate different layers of the colon.

There is currently no standard decellularization method. However, after extensive research on the current protocols for decellularization methods, decellularizing agents 2% sodium deoxycholate, 0.5% Triton X-100 in 2% SDS (SDS-Triton), and 1x trypsin/EDTA solution (0.05% trypsin)7,9 were chosen because they were amongst the most common decellularizing agents found in literature.

Methods

Preparation of Decellularization Reactions

Whole porcine intestines (Figure 1) were purchased from Ranch 99 supermarket in Albany, CA, and the colon was identified and separated from the small intestine. After the source colon was slit down its length, 30mmx30mm squares (containing both luminal and apical faces) were cut out and stored in a 1x PBS-penicillin/streptomycin bath. Three different decellularization agents were tested: 2% sodium deoxycholate, 0.5% Triton X-100 in 2% SDS (SDS-Triton), and 1x trypsin/EDTA solution (0.05% trypsin)7,9. Each solution was prepared with the reagents and with stock PBS + 1% penicillin/streptomycin. The PBS-penicillin/streptomycin maintained the pH of the decellularization agents and conferred antibacterial protection to each treated tissue.

Decellularization Reactions

Cut porcine colon tissue was placed in decellularization reagent, with each square of tissue immersed in 3 mL of decellularization reagent and allowed to shake at room temperature. There were two type of decellularization treatment and a control PBS +P/S solution treatment. The first decellularization reagent was Trypsin-EDTA. The second decellularization reagent was SDS-Triton. Each treatment was performed on the porcine colon tissue for 4 hours, and then the decellularized sample tissue was frozen down. Similarly, each treatment method was also performed on the tissue for 24 hours and then frozen.

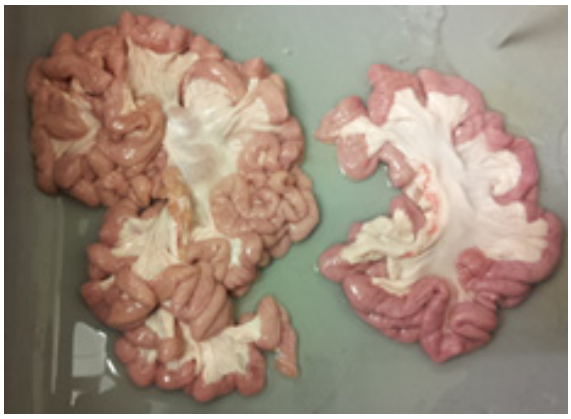

Figure 1: Porcine small intestine (left) and large intestine (right).

Recellularization Reactions

Caco-2 cells were cultured at low passage number on 10% FBS-DMEM. For recellularization, each decellularization-treated scaffold (one of triplicates) was submerged in 4 mL of Caco-2 cells at an initial seeding density of 4x104 cells/mL in 20% FBS-DMEM. Recellularization reactions were maintained in a CO2 incubator at 37°C in the liquid cell culture for 144 hours to allow for at least two complete 68-hour growth cycles for Caco-2 cells. Afterwards, scaffolds were washed and frozen in PBS+P/S for preservation and prior to imaging.

SEM imaging

SEM imaging was performed at the Biomolecular Nanotechnology Center (BNC), a QB3-affiliated laboratory that carries out various nanoscientific protocols and experiments for various groups affiliated with UC Berkeley. Small 25 square-milimeter samples were cut out from the native colon and the decellularized tissue immediately after the end of the reaction. These samples were allowed to dry before being coated with gold nanoparticles by low-vacuum sputter coating in order to provide an electrically conductive patina for SEM imaging. A wet sample would later be run, given the suspected effects of dehydration on structural and functional aspects of the scaffold at the nanoscale.

Hoeschst staining

A cryosectioned replicate of each completed decellularization reaction was immersed in PBS-penicillin/streptomycin buffer and few drops of the Hoechst staining reagent were added. The reaction was allowed to develop at room temperature in the darkness for twenty minutes before imaging.

NuMA staining

NuMA immunohistochemical staining was used to identify human nuclear marker (NuMA), a nuclear mitotic apparatus protein, as an indicator for human cell presence on the scaffold. Hoechst staining was used to identify DNA, as the dye binds to the minor groove at (preferentially) AT-rich sequences. Cryosectioned slices 10-um thin and prepared from 5x5 mm2 samples of post-recellularization scaffolds were adhered onto microscope slides with electrostatic surface charge, washed with 0.025% Triton X-100 in PBS, and blocked with 1% BSA in PBS. Slices were incubated with goat polyclonal anti-NuMa IgG primary antibody (Santa Cruz Biotechnology) at 1:100 dilution overnight at 4°C prior to 1% BSA in PBS washing and blocking. Bound primary antibody was probed with Alexa Fluor 488 donkey anti-goat IgG secondary antibody at 1:200 dilution and room temperature, which was conjugated to a fluorophore (excitation wavelength at 488 nm) that emits in the green spectrum. After fixation with 4% paraformaldehyde for 15 minutes, slices were treated with Hoechst stain at 1:2000 dilution and room temperature for 20 minutes prior to PBS washing. Slides were mounted onto a coverslip and imaged under three conditions at the same location: the white-light field to capture the slice contour, the DAPI filter to capture Hoechst-positive signal, and the methyl green filter to capture the NuMA-positive signal.

Results

Maximum decellularization of colon achieved using Trypsin/EDTA 24-hour wash

Hoechst staining was performed on the decellularized scaffolds (Figure 2), and imaging was performed at 20x magnification. Cells that remain on the scaffold stand out as small, pale-blue circular spots on the tissue (since DAPI staining highlights the nucleus). Large blue diffuse clouds in the tissue indicate non-specific binding of the indicator to components of the decellularized sample. Based on these Hoechst staining results, the short wash of SDS-Triton achieved the best decellularization. The number and size of stained cells in the Hoecsht staining of the Trypsin-EDTA short wash indicate inefficient decellularization. Even if decellularization did occur, there is a lot of residual DNA left over. As a matter of fact, the Trypsin-EDTA 24-hour wash was appears to have very similar results to the SDS-Triton Short wash. The SDS-Triton 24-hour wash has almost no evidence of cells. SDS and Triton X-100 are both powerful detergents that extensively removed cells and reduced the viability of any cells that remained on the ECM after the treatment. On the other hand,

trypsin is a protease that would sever the connection between cells and a scaffolding layer, facilitating the detachment of the

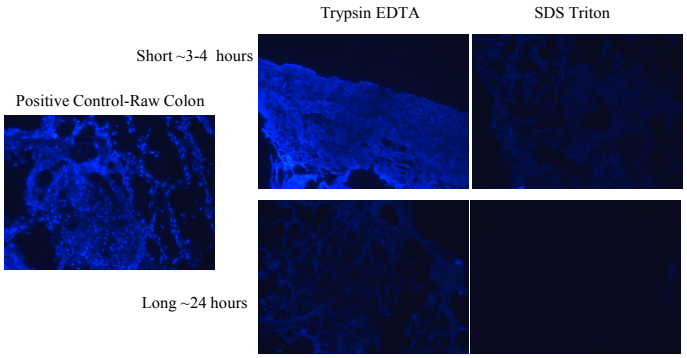

Figure 2: Hoechst stain results. Magnification is 20x.

cells from the scaffold. The 24-hour wash provided better decellularization outcomes than the 4-hour wash, suggesting that longer exposures to SDS-Triton result in greater decellularization outcomes. The Trypsin-EDTA short wash looks very similar to the positive control in Figure 2.

SEM imaging reveals topological properties of decellularized scaffold

Pairs of SEM images for each of the native lumen and the three successful decellularization reactions are given in Figure 3. The purpose of this experiment was to explore the effects of decellularization upon the treated construct's topology and

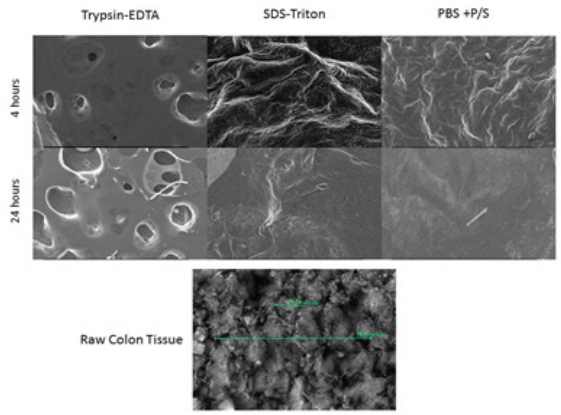

Figure 3: SEM imaging of decellularization reagent-treated samples at 200 nm.

constitution on the nanometer scales. Nanoscale investigation was important because few other methods provide as high-resolution view of the actual interactions between the scaffold and any straggling cells as does SEM. High resolution imaging allowed us to corroborate with greater detail the basis for our conclusions on what each decellularization agent does to its sample. SEM would allow us to understand what scaffolds from different decellularization reactions would actually look like.

Figure 3 illustrates the SEM imaging of the decellularized tissue and native control colon tissue. The native tissue exhibited a surface riddled with clusters, indicating the enterocytes that resided upon the surface of the lumen. Interspersed were short hair-like segments that, upon investigation, appeared to be fibrils that could serve chiefly structural properties in the scaffold. The SDS-Triton-treated tissue showed long, dark, deep grooves throughout the surface. However, the shape of the scaffold, along with the hair-like fragments, remained largely intact compared to those within the native sample. The SDS-Triton short decellularization treatment yielded preservation of the microvilli structure of the colon. Taken together, these results indicate that SDS-Triton serves as a chiefly cytotoxic agent, killing and dislodging enterocytes from the scaffold. The SDS-Triton largely spared the structures of the scaffolds because it does not chiefly function as a proteolytic agent and because the complicated cross-linking of the ECM renders it difficult to degrade. Both of the trypsin/EDTA treatments exhibited very different structural differences from the native tissue and the SDS-Triton-treated tissue. The trypsin/EDTA short wash resulted in smoothed, slightly fissured, and devoid of any hair-like segments found arcing out of cells in the tissue. The 24-hour wash was completely devoid of the intricate contour and texture characteristic of the scaffold in other samples. Taken together, this indicates that prolonged application of trypsin can efface some of the extracellular matrix for cells by breaking down the peptide components of the scaffold (as trypsin is a potent protease) and eroding the structures in which cells would reside. Furthermore, voids are evident in the Trypsin-EDTA treated tissue that indicates that the entire microvilli structure was destroyed by the Trypsin protease. The voids are from the submucosa, exposed upon removal of the upper layer (a major functional unit of the colon) during decellularization.

Scaffold of 24-hour washed trypsin/EDTA-treated and short-SDS Trion treated colon tissue experiences maximum recellularization

Figure 4 illustrates the recellularization of the decellularized

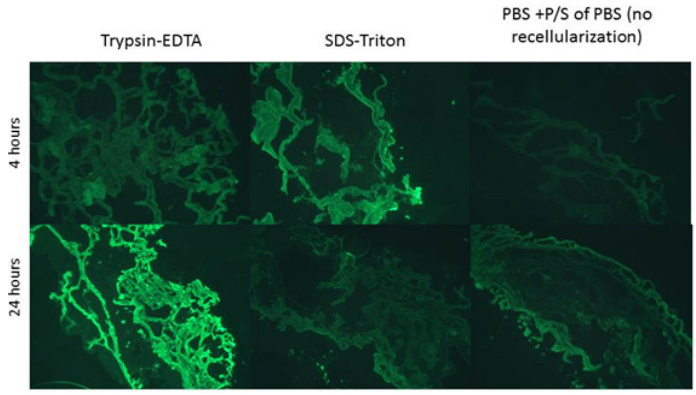

Figure 4: NuMA staining of decellularized tissue that was recellularized and control of NuMa staining on Normal Colon tissue in PBS decellularization treatment at 20x.

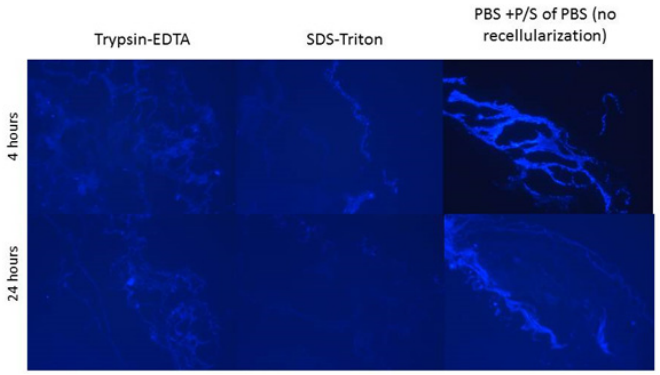

Figure 5: Hoechst staining of decellularized tissue which was recellularized and control of Hoechst staining on Normal Colon tissue in PBS decellularization treatment at 20x.

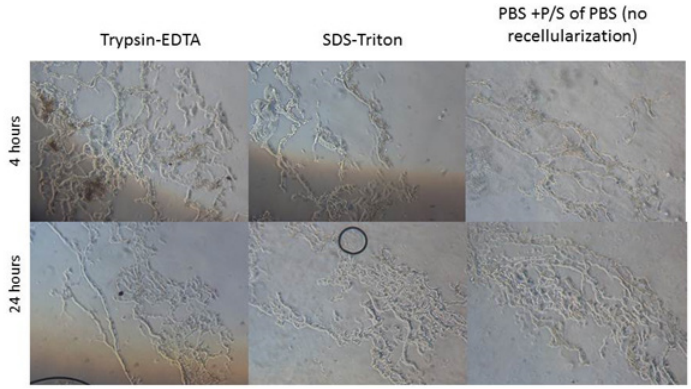

Figure 6: Bright Fields Views at 20x of Figure 4 and Figure 5.

tissue porcine scaffolds with Human Caco-2 cells, which display the enterocyte phenotype found in intestinal cells. NuMa was used to stain the human Caco-2 cells, because it is a human nuclear mark, and would help to differentiate any residual porcine cells that may have remained on the scaffolds after decellularization and wash treatment. SDS-Triton short decellularization treatment and Trypsin-EDTA 24-hour wash decellularization treatment revealed the best recellularization potential as shown in Figure 4. Figures 5 and 6 are images of the same cross sections except that Figure 5 stains for DNA content overall using Hoechst and Figure 6 is a bright field image. Different exposure times for images complicate interpretation of the results. However, given the difficulties in imaging the tissue and in uneven staining across each tissue slice, different exposures were necessary to ensure that each tissue was imaged clearly.

Discussion

We judged the effectiveness of decellularization based on two factors: the discharge of host enterocytes from the extracellular matrix, and the integrity of the matrix structure after treatment. Based on Hoescht imaging in Figure 2, the SDS-Triton wash was the most effective agent for decellularization; based on SEM imaging, the SDS-Triton short treatment was the most effective. Many other studies posit that SDS-Triton is better than trypsin/EDTA as a decellularization agent. While SDS-Triton does do a more effective job than trypsin/EDTA in decellularizing cells within a short time, trypsin/EDTA as a protease effectively clips the protein-cell junctions that anchor cells to the scaffold. Exposing the tissue to trypsin/EDTA for elongated periods of time degrades proteins in matrix, which can result in a less-than-optimal scaffold for recellularization if decellularization is carried out for long periods of time. In addition, trypsin/EDTA-treated scaffolds that are not washed properly can carry the risk of holding residual trypsin and deter recellularization due to lingering proteolytic effects of trypsin. Therefore, we've demonstrated that SDS-Triton is the more ideal decellularization treatment.

In comparing the results of recellularization, the scaffold from the 4-hour wash of the SDS-Triton was seeded with more cells than the other treatments, with the exception of Trypsin-EDTA 24-hour wash based on the NuMA stain results from Figure 4. A 4-hour wash with SDS-Triton resulted in less decellularization than a 24-hour treatment but allowed for more cellular presence on the tissue afterwards than the 24-hour wash. As a strong detergent, shorter application of SDS-Triton results in strong decellularization but also results in less erosion of the scaffold structure, since SDS solubilizes large protein aggregates. Thus, the shorter SDS-Triton application does not remove as many cells as the 24-hour application yet can preserve more of the structure to allow for new cell attachment and growth. The 4-hour SDS Triton allows for more efficient recellularization, while the 24-hour SDS Triton allows for more efficient decellularization. In addition, while Hoechst staining distinguishes the nuclei of the cells on the scaffold, it does not indicate if the cells' mitochondria are active and if, as a result, the cells are actively respiring.

We successfully carried out a pair of decellularization-recellularization reactions in sequence to engineer a luminal

graft for a human colon. Our project, a set of proof-of-concept experiments, demonstrated that the "cell-on-cell-off" workflow could be used to engineer some aspects of the human colon with reasonable effectiveness. We also showed that reagents used in decellularization were linked to the recellularization potentials of their scaffolds. Finally, we demonstrated the power of SEM imaging and NuMA staining assays in quantifying and describing the nature of decellularized and recellularized scaffolds. Our strategy is a first novel step to whole-colon fabrication. We've shown that selecting an optimal scaffold for colon regeneration involves: 1) achieving a balance of good decellularization based on removal of cells and DNA content 2) Preservation of colon microstructure and microvilli structure upon decellularization and 3) Potential for recellularization. The SDS-Triton short treatment reveals the most optimal balance of all three of the above conditions.

This idea of whole organ colon engineering is important for neonatal patients, given bowel obstruction disease is a severe issue in newborn children. The current treatment involves resection of the colon, and stretching of the intestine. Using our technology, with more advances in tissue engineering, we could use engineering biological tissue to implant a functional colon.

Acknowledgments

Zoey Huang, Phin-Peng Lee, Hayley J. Lam, PhD, and Song Li, PhD, provided mentorship. Naima Azgui and Paul Lum of the BNC provided assistance with SEM. Norman Bae and Noah Goldman assisted with initial work on the project. The authors would also like to thank the reviewers for their comments on the manuscript. This project was funded by the Department of Bioengineering at UC Berkeley.

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