Design and use of a rotating bioreactor for the culture of laryngeal transplants

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Abstract

Background: Patients requiring laryngeal transplants post-laryngectomy are faced with possible immune rejection and difficulty with donor matching, resulting in procedure with a very low efficacy. It has been shown that organs can be decellularized, leaving behind the extracellular matrix, and then recellularized with a patient's stem cells to avoid immune rejection upon transplantation. This presents an opportunity for a larynx to be decellularized and recellularized for use in transplants.

Methods: The bioreactor was fabricated using polycarbonate plastic with a CNC mill. To decellularize the tissue, the larynx is perfused with sodium dodecyl sulfate (SDS) and examined using DAPI. Canine epithelial cells are cultured and perfused through the decellularized matrix. Progressive biopsies and hematoxylin and eosin stains are used to analyze the tissue.

Results: The tissue was successfully decellularized using SDS perfused through the vasculature (speculative). Recellularization results show full adhesion to the matrix and no cell death (speculative).

Discussion: A concentration of 1% SDS successfully decellularized the tissue in less than a week, and any concentrations higher than this proved to be unnecessary. Directly seeding fibroblasts onto the laryngeal tissue and culturing for six hours gave the best adhesion and minimized cell death.

Conclusions: The novel bioreactor presented successfully facilitates both decellularization and recellularization of a canine larynx. Next, the process will be optimized for human laryngeal tissue.

Keywords: Laryngeal, bioreactor, recellularization, decellularization, perfusion, fluid dynamics modeling

Background

The need for organ replacement has grown in prominence over the last fifteen years. Wait lists for organs often exceed the donor rate tenfold (1). Laryngeal cancer affects 136,000 individuals worldwide each year, and a majority of these patients require a partial or total removal of this organ to stop the spread of cancer (2). After surgery, patients may be mute or may have to breathe through a stoma which can be problematic as it requires changes in daily activities such as speaking, showering, and sleeping (3). Laryngeal transplants can eliminate the need for a stoma, but at present there have only been

two successful cases of full laryngeal transplant due to a complicated surgery and anatomical complexities making donor matching difficult (2, 4, 5). Additionally, patients that receive an allograft larynx transplant must live with the risk of tumor recurrence, metastasis, and multi-infection as well as face lifelong immunosuppression. Researchers have suggested that decreasing the immune response of the implantation by using the patient's own mesenchymal stem cells (MSC) to generate an organ could clear up many of the problems currently associated with laryngeal allografts (2). Decellularizing the donor organ using detergents leaves behind a viable extracellular matrix (ECM) that can then be repopulated with the patient's MSCs. This process cannot be controlled *in vivo*, so a bioreactor must be available to construct an *ex vivo* environment that encourages cell proliferation and differentiation.

This method has been applied to a variety of whole organs, including the heart (6), the lungs (7), and the liver (8). Additionally, parts of the larynx have been made using this method as well. Laryngeal vocal folds have been grown in a bioreactor and have been shown to properly be able to withstand stresses similar to those seen in a biological environment (9). Finally, a trachea, which shares similar anatomical features to the larynx, has been grown *in vitro* using this method and implanted in a patient (10). We present a novel bioreactor that facilitates the decellularization of a donor larynx and recellularization of the extracellular matrix with MSCs by rotation of the larynx in media and perfusion of detergents or media through the inner lumen and vasculature. The bioreactor minimizes the amount of media needed, is autoclavable, and allows automation of pump flow and larynx rotation.

Methods

Bioreactor Materials and Fabrication

The bioreactor is fabricated out of LEXAN polycarbonate (Grainger), besides the sealant and screws. Polycarbonate is one of the strongest thermoplastic polymers, can be machined easily, and is inert when contacting the SDS media. The other materials include number 8 machine screws (Dorn True Value) to secure the bottom piece of the bioreactor, a 0.5-inch rubber stopper for draining the media, and two rubber O-rings (Parker).

The bioreactor walls were cut out of polycarbonate using a band saw to the external dimensions of 15x15x25cm. The stepper motor housing pieces were also cut in similar fashion. The cage was fabricated using a CNC mill and the bars were cut to size using a band saw. The holes for the cage and the bars connecting the cage to the walls were drilled with a mill. The stepper motor was connected to the cage via a polycarbonate rod and a set-screw. The hole for the drain was cut on the bottom of the bioreactor. The bioreactor was assembled with silicone sealant.

Pumps and Electronics

The system also includes two peristaltic pumps (Langer Instruments). The bioreactor cage is moved using a stepper motor that is controlled by an Arduino Uno (SparkFun) and an EasyDriver (SparkFun). The software used with the Arduino is called Arduino IDE. The electronics were soldered together. The system was tested to verify that the electronics and pumps functioned according to the manufacturers'' specifications.

Setup of the System

The pumps should be taken out of the box and placed near the bioreactor. The larynx should be strapped into the cage. The cage needs to be put inside of the bioreactor and the support rods attached to the cage. The stepper motor needs to be attached to the rod and placed in the motor housing. The EasyDriver needs to be wired to the Arduino Uno and the pumps need to the wired to the peristaltic pumps. Finally the power cords for the stepper motor and Arduino should be plugged in.

Decellularization

- A fresh canine larynx is secured with four sutures (two superiorly, two anteriorly) to the cage portion of the device. A known concentration of sodium dodecyl sulfate (SDS) solution is perfused through the interior of the tissue, as well as through the vasculature for a specified

period of time. The void volume of the bioreactor is then filled with a known concentration of SDS solution. The larynx, attached to the device, is then turned a specified amount, pausing for a specified amount of time between each rotation, for the duration of the testing. The specific conditions of each test will be outlined in the results section below. Immunohistochemistry will be used to verify the presence of extracellular elements. Additionally, DAPI stains will be used to detect residual nuclei.

Recellularization

- Canine epithelial cells will be isolated and cultured, with a method that is yet to be determined.
- A decellularized canine larynx (method of decellularization TBD), as verified through (method of verifying decellularization TBD) will be secured with sutures to the cage portion of the device. An aqueous media composed of the aforementioned canine fibroblasts will be seeded directly onto the decellularized scaffold. The seeded scaffold will then be submerged in media (composition TBD) and held static for a period of time, followed by a period of tissue rotation to allow seeded cells variable exposure to air and media, as well as variable media perfusion velocities (specifics TBD),
- During recellularization, progressive biopsies will be taken of the tissue and fixed. Hematoxylin and eosin (H&E) stains will be conducted on these biopsies to assess cellular adhesion, proliferation, and differentiation.

Modeling

- Fluid dynamics modeling serves to model the fluid flow in the bioreactor. The program ANSYS would be used to test the fluid profile in the bioreactor because it changes with the movement of the cage and can alter how the fluid interacts with the tissue. Also because the laryngeal tissue is being exposed to air, the model should ensure that the entirety of the tissue is interacting with the fluid as well as air. From this model we would be able to assess the optimal turning speed and turning distance of the cage.

Results and Discussion

Decellularization

- Here the team will outline specifics of each progressive trial, including why variables were altered. This will also be accompanied by a table displaying the different experimental conditions
- Differences according to changes in experimental procedure will be discussed, and the team will hypothesize why these changes occurred. Particularly, the team will seek to point out which experimental conditions yielded the optimal decellularization of the tissue and why this occurred in this fashion. Attention will be given to procedures that yield the fastest decellularization as well as procedures that yield the most complete decellularization.
- Figures in this section will include comparative images of the decellularized tissue, as well as comparative images of histological stains. These figures will demonstrate the differences between experimental procedures and will, ideally, quickly and pictorially show readers the difference between a poor and excellent decellularization techniques.
- The team expects that a 1% SDS solution perfused through the organ for 120 hours, accompanied by a tissue rotation of 180 degrees every 15-30 seconds will allow the best decellularization conditions. This is because it allows adequate time for complete decellularization with a low SDS concentration, so that extracellular tissue will not be destroyed. Additionally, this rotation profile gives adequate mixing and variable exposure to the media bath and oxygen.

Recellularization

- Here the team will outline the progression of recellularization trials, highlighting the successes and failures of each trial and hypothesize why these changes occurred with the corresponding variations in experimental procedure. This section will also feature a table outlining all of the experimental procedures used and a summary of the results
- The team will also critically examine the evaluation techniques used to survey cell proliferation,

adhesion, and differentiation, and comment on the efficacy of the use of these techniques in the production of a viable and reproducible method for seeding and culturing a whole laryngeal organ.

- The team will draw conclusions based on experimental evidence as well as modeling evidence (discussed below) as to the current best method for seeding and culturing whole-organ laryngeal tissue and will use this data to suggest future directions for this facet research.
- Figures will be crucial to the understanding and conveying of the data acquired in this research. Photographs of the recellularized tissues will be included with detailed captions pointing out the differences in recellularized tissue structure. Additionally, pictorial representations of H&E stains will be crucial in conveying to non-engineering readers the efficacy of the device. Finally, any non-pictorial based assays used will be represented in graphical form and will be explained with detailed captions.
- The team expects, based on prior research conducted on the recellularization of hollow organs (11, 12), that direct seeding of fibroblasts, followed by a period of stagnant placement of the seeded organ in media for at least six hours will result in the best adhesion of cells to the decellularized tissue. Research has also shown that, after this initial period of stagnancy, rotation of the organ at a constant rate of 1.5 revolutions per hour with perfusion of media at a low rate through the lumen and vasculature of the tissue results in the best cell proliferation and minimal undesired apoptosis. We expect the results from this study to show a similar successful recellularization technique.

Modeling

We are performing the modeling of the bioreactor because it will give us a better indication of how to program our motor. This will provide better decellularized tissues before recellularization. It will also allow us to design the experiment for optimal recellularization. This insight will lead to further work being done not only on laryngeal tissue but also other tissues. We expect to be able to give our clients an

optimal process for our bioreactor, and possibly give our product to them already programmed.

Future Work

While it is difficult to speculate, at this time, what future work will entail after the completion of the research outlined here, we expect that the following steps will need to be taken after the completion of this semester:

- **The continued improvement of hollow-organ bioreactor design:** there are expected to still be improvements to be made to the design of the bioreactor that are not foreseeable at this point. In order to ensure the continued improvement of this design, the team will conduct an ergonomics survey with the clients in order to outline future design improvements that can be made.
- **The streamlining of the decellularization procedure for hollow organs**: Once a procedure has been determined that will appropriately decellularize the organ, future work involving decellularization should involve optimizing the procedure and minimizing media used for scale-up purposes. At this time, we hypothesize that this will involve the incorporation of a media filtration and recycling system.
- **Continued research into recellularization of organs composed of multiple distinct tissue types:** It is highly unlikely that, in eleven weeks' time, recellularization of an organ as complex as a larynx can be successfully and completely recellularized. Therefore, continued work will be needed in order to determine how to adequately recellularize this organ. This work could involve the incorporation of other cell types, alteration of media used, alteration of flow profiles used, or many other alterations of experimental procedure.
- **The development of a more quantifiable model for whole organ decellularization and recellularization procedures:** For future research as well as scale-up, it will be vital to fully understand and characterize the decellularization and recellularization profiles of the organ. The use of a model such as this will allow prediction of how changes in experimental procedure will

affect cellular activity in decellularization and recellularization procedures which will, in turn, allow for safer, more efficient research in this area.

- **The investigation of a more widespread implementation of the app-based bioreactor programming system designed here:** The use of an app-based system for bioreactor control is, based on our limited research, a relatively novel concept; only one such commercial system is known to exist at this time (13). However, the use of a user-intuitive application for controlling and manipulating a bioreactor environment is necessary, especially for users who may not be familiar with programming and engineering applications. Therefore, the use of this app, as well as its implications for use on multiple bioreactor types, should be continued.

List of abbreviations

SDS – sodium dodecyl sulfate

MSC – mesenchymal stem cell

ECM – extracellular matrix

H&E – hematoxylin and eosin

Competing interests

The researchers have no competing interests to report.

Acknowledgements

We would like to thank our advisor, Dr. Tracy Puccinelli, for all of her assistance with the project. Additionally, the team extends our thanks to Dr. Nathan Welham and Dr. Yutaka Toya for proposing and facilitating the project, and to Dr. Seung Won Lee for working extensively with the bioreactor. Finally, our thanks to the University of Wisconsin - Madison students, Brett Napiwocki, Taylor Milne, Armand Grabowski, Sara Schmitz, and Ben Smith, for their previous contributions to the project.

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Figure Captions

Figure 1: A: Complete larynx extracted from a human donor. **B:** Complete canine larynx partially decellularized. This larynx was placed in 1% SDS solution for 96 hours. Note the lack of redness and the whiteness of the tissue. This is indicative of decellularized tissue.